

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷: C12N 15/00, 15/20, C07K 17/08, 14/555, 14/475	A1	(11) International Publication Number: WO 00/42175 (43) International Publication Date: 20 July 2000 (20.07.00)
(21) International Application Number: PCT/US00/00931 (22) International Filing Date: 14 January 2000 (14.01.00) (30) Priority Data: 60/116,041 14 January 1999 (14.01.99) US (71) Applicant (for all designated States except US): BOLDER BIOTECHNOLOGY INC. [US/US]; 678 West Willow Street, Louisville, CO 80027 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): COX, George, N. [US/US]; 678 West Willow St., Louisville, CO 80027 (US). DOHERTY, Daniel, H. [US/US]; 719 Ithaca Drive, Boulder, CO 80203 (US). ROSENDAHL, Mary, S. [US/US]; 310 Fairplay Ave., Broomfield, CO 80020 (US). (74) Agents: BROWN, Theresa, A. et al.; Sheridan Ross P.C., Suite 1200, 1560 Broadway, Denver, CO 80202-5141 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHODS FOR MAKING PROTEINS CONTAINING FREE CYSTEINE RESIDUES (57) Abstract The present invention relates to novel methods of making soluble proteins having free cysteines in which a host cell is exposed to a cysteine blocking agent. The soluble proteins produced by the methods can then be modified to increase their effectiveness. Such modifications include attaching a PEG moiety to form pegylated proteins.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS FOR MAKING PROTEINS CONTAINING FREE CYSTEINE RESIDUES

Field of the Invention

5 The present invention relates generally to methods of making proteins and more specifically to recombinant proteins containing a “free” cysteine residue that does not form a disulfide bond.

Background of the Invention

10 There is considerable interest on the part of patients and healthcare providers in the development of low cost, long-acting, “user-friendly” protein therapeutics. Proteins are expensive to manufacture and unlike conventional small molecule drugs, are usually not readily absorbed by the body. Moreover they are digested if taken orally. Therefore, proteins must typically be administered by injection. After injection most proteins are cleared rapidly from the body, necessitating frequent, often daily, injections. Patients dislike injections, which leads to reduced compliance and reduced
15 drug efficacy. Some proteins such as erythropoietin (EPO) are effective when administered less often (three times per week for EPO) but this is due to the fact that the proteins are glycosylated. Glycosylation requires that the recombinant proteins be manufactured using mammalian cell expression systems, which is expensive and increases the cost of protein pharmaceuticals.

20 Thus, there is a strong need to develop protein delivery technologies that lower the costs of protein therapeutics to patients and healthcare providers. One solution to this problem is the development of methods to prolong the circulating half-lives of protein therapeutics in the body so that the proteins do not have to be injected frequently. This solution also satisfies the needs and desires of patients for protein therapeutics that are “user-friendly”, i.e., protein therapeutics that do not require frequent injections.

25 Covalent modification of proteins with polyethylene glycol (PEG) has proven to be a useful method to extend the circulating half-lives of proteins in the body (Abuchowski et al., 1984; Hershfield, 1987; Meyers et al., 1991). Covalent attachment of PEG to a protein increases the protein’s effective size and reduces its rate of clearance from the body. PEGs are commercially available in several sizes, allowing the circulating half-lives of PEG-modified proteins to be tailored
30 for individual indications through use of different size PEGs. Other documented *in vivo* benefits of PEG modification are an increase in protein solubility, stability (possibly due to protection of the protein from proteases) and a decrease in protein immunogenicity (Katre et al., 1987; Katre, 1990).

 One known method for PEGylating proteins uses compounds such as N-hydroxy succinimide (NHS)-PEG to attach PEG to free amines, typically at lysine residues or at the N-terminal amino acid.
35 A major limitation of this approach is that proteins typically contain several lysines, in addition to the N-terminal amino acid, and the PEG moiety attaches to the protein non-specifically at any of the available free amines, resulting in a heterogeneous product mixture. Many NHS-PEGylated proteins are unsuitable for commercial use because of low specific activities and heterogeneity. Inactivation results from covalent modification of one or more lysine residues or the N-terminal amino acid

required for biological activity or from covalent attachment of the PEG moiety near the active site of the protein.

Of particular relevance to this application is the finding that modification of human growth hormone (hGH) with amine-reactive reagents, including NHS-PEG reagents, reduces biological activity of the protein by more than 10-fold (Teh and Chapman, 1988; Clark et al., 1996). GH is a 22 kDa protein secreted by the pituitary gland. GH stimulates metabolism of bone, cartilage and muscle and is the body's primary hormone for stimulating somatic growth during childhood. Recombinant human GH (rhGH) is used to treat short stature resulting from GH inadequacy, Turner's Syndrome and renal failure in children. GH is not glycosylated and is fully active when produced in bacteria. The protein has a short *in vivo* half-life and must be administered by daily subcutaneous injection for maximum effectiveness (MacGillivray et al., 1996).

There is considerable interest in the development of long-acting forms of hGH. Attempts to create long-acting forms of hGH by PEGylating the protein with amine-reactive PEG reagents have met with limited success due to significant reductions in bioactivity upon PEGylation. Further, the protein becomes PEGylated at multiple sites (Clark et al., 1996). hGH contains nine lysines, in addition to the N-terminal amino acid. Certain of these lysines are located in regions of the protein known to be critical for receptor binding (Cunningham et al., 1989; Cunningham and Wells, 1989). Modification of these lysine residues significantly reduces receptor binding and bioactivity of the protein (de la Llosa et al., 1985; Martal et al., 1985; Teh and Chapman, 1988; Cunningham and Wells, 1989). hGH is readily modified by NHS-PEG reagents, but biological activity of the NHS-PEG protein is severely compromised, amounting to only 1% of wild type GH biological activity for a GH protein modified with five 5kDa PEG molecules (Clark et al., 1996). The EC₅₀ for this multiply PEGylated GH protein is 440 ng/ml or approximately 20 nM (Clark et al., 1996). In addition to possessing significantly reduced biological activity, NHS-PEG-hGH is very heterogeneous due to different numbers of PEG molecules attached to the protein and at different amino acid residues, which has an impact on its usefulness as a potential therapeutic. Clark et al. (1996) showed that the circulating half-life of NHS-PEG-hGH in animals is significantly prolonged relative to non-modified GH. Despite possessing significantly reduced *in vitro* biological activity, NHS-PEG-hGH was effective and could be administered less often than non-modified hGH in a rat GH-deficiency model (Clark et al., 1996). However, high doses of NHS-PEG-hGH (60-180 µg per injection per rat) were required for efficacy in the animal models due to the low specific activity of the modified protein. There is a clear need for better methods to create PEGylated hGH proteins that retain greater bioactivity. There also is a need to develop methods for PEGylating hGH in a way that creates a homogeneous PEG-hGH product.

Biological activities of several other commercially important proteins are significantly reduced by amine-reactive PEG reagents. EPO contains several lysine residues that are critical for bioactivity of the protein (Boissel et al., 1993; Matthews et al., 1996) and modification of lysine residues in EPO results in near complete loss of biological activity (Wojchowski and Caslake, 1989). Covalent modification of alpha-interferon-2 with amine-reactive PEGs results in 40-75% loss of

bioactivity (Goodson and Katre, 1990; Karasiewicz et al., 1995). Loss of biological activity is greatest with large (e.g., 10 kDa) PEGs (Karasiewicz et al., 1995). Covalent modification of G-CSF with amine-reactive PEGs results in greater than 60% loss of bioactivity (Tanaka et al., 1991). Extensive modification of IL-2 with amine-reactive PEGs results in greater than 90% loss of bioactivity
5 (Goodson and Katre, 1990).

A second known method for PEGylating proteins covalently attaches PEG to cysteine residues using cysteine-reactive PEGs. A number of highly specific, cysteine-reactive PEGs with different reactive groups (e.g., maleimide, vinylsulfone) and different size PEGs (2-40 kDa) are commercially available. At neutral pH, these PEG reagents selectively attach to "free" cysteine
10 residues, i.e., cysteine residues not involved in disulfide bonds. Cysteine residues in most proteins participate in disulfide bonds and are not available for PEGylation using cysteine-reactive PEGs. Through *in vitro* mutagenesis using recombinant DNA techniques, additional cysteine residues can be introduced anywhere into the protein. The newly added "free" cysteines can serve as sites for the specific attachment of a PEG molecule using cysteine-reactive PEGs. The added cysteine residue can
15 be a substitution for an existing amino acid in a protein, added preceding the amino-terminus of the protein or after the carboxy-terminus of the protein, or inserted between two amino acids in the protein. Alternatively, one of two cysteines involved in a native disulfide bond may be deleted or substituted with another amino acid, leaving a native cysteine (the cysteine residue in the protein that normally would form a disulfide bond with the deleted or substituted cysteine residue) free and
20 available for chemical modification. Preferably the amino acid substituted for the cysteine would be a neutral amino acid such as serine or alanine. Growth hormone has two disulfide bonds that can be reduced and alkylated with iodoacetimide without impairing biological activity (Bewley et al., (1969). Each of the four cysteines would be reasonable targets for deletion or substitution by another amino acid.

25 Several naturally-occurring proteins are known to contain one or more "free" cysteine residues. Examples of such naturally-occurring proteins include human Interleukin (IL)-2, beta interferon (Mark et al., 1984), G-CSF (Lu et al., 1989) and basic fibroblast growth factor (Thompson, 1992). IL-2, G-CSF and beta interferon contain an odd number of cysteine residues, whereas basic fibroblast growth factor contains an even number of cysteine residues.

30 However, expression of recombinant proteins containing free cysteine residues has been problematic due to reactivity of the free sulfhydryl at physiological conditions. Several recombinant proteins containing free cysteines have been expressed as intracellular proteins in bacteria such as *E. coli*. Examples include natural proteins such as IL-2, beta interferon, G-CSF, basic fibroblast growth factor and engineered cysteine muteins of IL-2 (Goodson and Katre, 1990), IL-3 (Shaw et al., 1992),
35 Tumor Necrosis Factor Binding Protein (Tuma et al., 1995), IGF-I (Cox and McDermott, 1994), IGFBP-1 (Van Den Berg et al., 1997) and protease nexin and related proteins (Braxton, 1998). All of these proteins were insoluble when expressed intracellularly in bacteria. The insoluble proteins could be refolded into their native conformations by performing a series of denaturation, reduction and refolding procedures. These steps add time and cost to the manufacturing process for producing the

proteins in bacteria. Improved stability and yields of IL-2 (Mark et al., 1985) and beta interferon (DeChiara et al., 1986) have been obtained by substituting another amino acid, e.g., serine, for the free cysteine residue. It would be preferable to express the recombinant proteins in a soluble, biologically active form to eliminate these extra steps.

5 One known method of expressing soluble recombinant proteins in bacteria is to secrete them into the periplasmic space or into the media. It is known that certain recombinant proteins such as GH are expressed in a soluble active form when they are secreted into the *E. coli* periplasm, whereas they are insoluble when expressed intracellularly in *E. coli*. Secretion is achieved by fusing DNA sequences encoding growth hormone or other proteins of interest to DNA sequences encoding
10 bacterial signal sequences such as those derived from the stII (Fujimoto et al., 1988) and ompA proteins (Ghrayeb et al., 1984). Secretion of recombinant proteins in bacteria is desirable because the natural N-terminus of the recombinant protein can be maintained. Intracellular expression of recombinant proteins requires that an N-terminal methionine be present at the amino-terminus of the recombinant protein. Methionine is not normally present at the amino-terminus of the mature forms
15 of many human proteins. For example, the amino-terminal amino acid of the mature form of human growth hormone is phenylalanine. An amino-terminal methionine must be added to the amino-terminus of a recombinant protein, if a methionine is not present at this position, in order for the protein to be expressed efficiently in bacteria. Typically addition of the amino-terminal methionine is accomplished by adding an ATG methionine codon preceding the DNA sequence encoding the
20 recombinant protein. The added N-terminal methionine often is not removed from the recombinant protein, particularly if the recombinant protein is insoluble. Such is the case with hGH, where the N-terminal methionine is not removed when the protein is expressed intracellularly in *E. coli*. The added N-terminal methionine creates a "non-natural" protein that potentially can stimulate an immune response in a human. In contrast, there is no added methionine on hGH that is secreted into
25 the periplasmic space using stII (Chang et al., 1987) or ompA (Cheah et al., 1994) signal sequences; the recombinant protein begins with the native amino-terminal amino acid phenylalanine. The native hGH protein sequence is maintained because of bacterial enzymes that cleave the stII-hGH protein (or ompA-hGH protein) between the stII (or ompA) signal sequence and the start of the mature hGH protein. While the periplasmic space is believed to be an oxidizing environment that should promote
30 disulfide bond formation, coexpression of protein disulfide isomerase with bovine pancreatic trypsin inhibitor resulted in a six-fold increase in the yield of correctly folded protein from the *E. coli* periplasm (Ostermeier et al., (1996). This result would suggest that periplasmic protein folding can at times be inefficient and is in need of improvement for large scale protein production.

hGH has four cysteines that form two disulfides. hGH can be secreted into the *E. coli*
35 periplasm using stII or ompA signal sequences. The secreted protein is soluble and biologically active (Hsiung et al., 1986). The predominant secreted form of hGH is a monomer with an apparent molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of 22 kDa. Recombinant hGH can be isolated from the periplasmic space by using an osmotic shock procedure (Koshland and Botstein, 1980), which preferentially releases periplasmic, but not

intracellular, proteins into the osmotic shock buffer. The released hGH protein is then purified by column chromatography ((Hsiung et al., 1986).

When similar procedures were attempted to secrete hGH variants containing a free cysteine residue (five cysteines; 2N+1), it was discovered that the recombinant hGH variants formed multimers and aggregates when isolated using standard osmotic shock and purification procedures developed for hGH. Very little of the monomeric hGH variant proteins could be detected by non-reduced SDS-PAGE in the osmotic shock lysates or during purification of the proteins by column chromatography.

Alpha interferon (IFN- α 2) also contains four cysteine residues that form two disulfide bonds. IFN- α 2 can be secreted into the E. coli periplasm using the stII signal sequence (Voss et al., 1994). The secreted protein is soluble and biologically active (Voss et al., 1994). The predominant secreted form of IFN- α 2 is a monomer with an apparent molecular weight by SDS-PAGE of 19 kDa. Secreted recombinant IFN- α 2 can be purified by column chromatography (Voss et al., 1994).

When similar procedures were attempted to secrete IFN- α 2 variants containing a free cysteine residue (five cysteines; 2N+1), it was discovered that the recombinant IFN- α 2 variants formed multimers and aggregates when isolated using standard purification procedures developed for IFN- α 2. The IFN- α 2 variants eluted from the columns very differently than IFN- α 2 and very little of the monomeric IFN- α 2 variant proteins could be purified using column chromatography procedures developed for IFN- α 2.

An alternative method to synthesizing a protein containing a free cysteine residue is to introduce a thiol group into a protein post-translationally via a chemical reaction with succinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (LC-SPDP, commercially available from Pierce Chemical Company). LC-SPDP reacts with lysine residues to create a free sulfhydryl group. Chemically cross-linked dimeric EPO was prepared using this reagent in conjunction with a maleimide protein modifying reagent (Sytkowsk et al., 1998). A heterologous mixture of chemically cross-linked EPO proteins was recovered after purification due to non-specific modification of the various lysine residues in EPO. Enhanced pharmacokinetics and *in vivo* potency of the chemically cross-linked EPO proteins were observed.

Another method that has been used to increase the size of a protein and improve its *in vivo* potency involves dimerization of the protein using chemical crosslinking reagents. GH is thought to transduce a cellular signal by cross-linking two GH receptors. A GH-GH dimer might facilitate enhanced receptor dimerization and subsequent amplification of the intracellular signal.

Chemically cross-linked dimeric hGH proteins have been described by Mockridge et al. (1998). Using a water soluble cross-linking reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), GH was randomly derivatized to give predominantly amide-linked dimers but also amide-linked multimers, depending on the concentration of EDC reagent used. While an increase in *in vivo* potency was observed, the final protein preparation was heterogeneous due to non-specific reaction of the EDC reagent with various amino acids in the protein, including lysine, aspartic acid and glutamic acid residues and the amino- and carboxy-termini. Injection of such a preparation into humans would be undesirable due to the toxic nature of EDC, potential immunogenic response to the

unnatural amide bond formed between the proteins. Generating consistent batches of a purified protein also would be difficult at the manufacturing scale.

Therefore, despite considerable effort, a need still exists for a process for generating homogeneous preparations of long acting recombinant proteins by enhancement of protein molecular weight. A need also for methods that allow secretion and recovery of recombinant proteins containing free cysteine residues in high yield. The present invention satisfies these needs and provides related advantages as well.

Summary of the Invention

10 The present invention relates to methods for obtaining a soluble protein having a free cysteine. The methods are generally accomplished by obtaining a host cell capable of expressing the soluble protein, exposing the host cell to a cysteine blocking agent, and isolating the soluble protein from the host cell. In one embodiment in which the protein is not secreted into the media by the host cell, the host cell is disrupted in the presence of the cysteine blocking agent and the soluble protein is
15 isolated or purified from the soluble fraction of the disrupted host cell. In another embodiment in which the soluble protein is secreted by the host cell into the media, the host cell is exposed to the cysteine blocking agent before, during or after synthesis of the soluble protein by the host cell.

Suitable host cells include bacteria, yeast, insect or mammalian cells. Preferably, the host cell is a bacterial cell, particularly *E.coli*.

20 Preferably, the soluble protein produced by the methods of the present invention are recombinant proteins, especially cysteine variants or muteins of a protein. The methods are useful for producing proteins including, without limitation, human growth hormone, EPO and interferon, especially alpha interferon, their derivatives or antagonists. Other proteins include members of the TGF-beta superfamily, platelet derived growth factor-A, platelet derived growth factor-B, nerve
25 growth factor, brain derived neurotrophic factor, neurotrophin-3, neurotrophin-4, vascular endothelial growth factor, or a derivative or an antagonist thereof. Cysteine muteins of heavy or light chain of an immunoglobulin or a derivative thereof are also contemplated.

Useful cysteine blocking agents include any thiol-reactive compound, including for example, cystine, cystamine, dithioglycolic acid, oxidized glutathione, iodine, hydrogen peroxide,
30 dihydroascorbic acid, tetrathionate, O-iodosobenzoate or oxygen in the presence of a metal ion.

The present methods further include various methods of attaching a PEG moiety to the soluble protein to form pegylated proteins in which the PEG moiety is attached to the soluble protein through the free cysteine. Higher order multimeric proteins involving the coupling of two or more of the soluble proteins are also within the present invention.

35 The present invention further includes the soluble proteins and their derivatives, including pegylated proteins, made by the methods disclosed herein. Such pegylated proteins include monopegylated hGh, EPO and alpha interferon.

The present invention also provides methods for pegylating the soluble proteins obtained by the methods described herein. Such methods include purifying the protein, reducing at least partially

the protein with a disulfide-reducing agent and exposing the protein to a cysteine-reactive moiety. Optionally, the modified cysteine protein can be isolated from unmodified protein.

Methods of treating conditions treatable by growth hormone, EPO and alpha interferon are also within the present invention. The soluble proteins or their derivatives, including pegylated derivatives, are administered to patients suffering from conditions in which known growth hormone, EPO or alpha interferon is effective.

Brief Description of the Figure

Figure 1 is a diagram of mutagenesis by overlap extension in which two separate fragments are amplified from a target DNA segment.

Description of the Invention

The present invention provides novel methods of obtaining proteins having free cysteine residues. The invention further provides novel proteins, particularly recombinant proteins, produced by these novel methods as well as derivatives of such recombinant proteins. The novel methods for preparing such proteins are generally accomplished by:

- (a) obtaining a host cell capable of expressing a protein having a free cysteine;
- (b) exposing the host cell to a cysteine blocking agent; and
- (c) isolating the protein from the host cell.

In one embodiment, the methods include the steps of disrupting the host cell in the presence of the cysteine blocking agent, followed by isolating the protein from the soluble fraction of the disrupted cell.

In a further embodiment in which the proteins are secreted into the media by prokaryotic or eukaryotic host cells, the methods include the steps of:

- (a) obtaining a host cell capable of expressing a protein having a free cysteine;
- (b) exposing the host cell to a cysteine blocking agent during synthesis, or after synthesis but prior to purification, of the protein having a free cysteine residue; and
- (d) isolating the protein from other cellular components in the media.

As identified above, the first step in these methods is to obtain a host cell capable of expressing a protein having a free cysteine residue. Suitable host cells can be prokaryotic or eukaryotic. Examples of appropriate host cells that can be used to express recombinant proteins include bacteria, yeast, insect and mammalian cells. Bacteria cells are particularly useful, especially *E.coli*.

As used herein, the term "protein having a free cysteine residue" means any natural or recombinant protein peptide containing $2N+1$ cysteine residues, where N can be 0 or any integer, and proteins or peptides containing $2N$ cysteines, where two or more of the cysteines do not normally participate in a disulfide bond. Thus, the methods of the present invention are useful in enhancing the expression, recovery and purification of any protein or peptide having a free cysteine, particularly

cysteine added variant recombinant proteins (referred to herein as "cysteine muteins" or "cysteine variants") having one or more free cysteines and/or having 2 or more cysteines that naturally form a disulfide bond. Although the expression, recovery and purification of a natural protein having a free cysteine expressed by its natural host cell can be enhanced by the methods of the present invention, the description herein predominantly refers to recombinant proteins for illustrative purposes only. In addition, the proteins can be derived from any animal species including human, companion animals and farm animals.

Accordingly, the present invention encompasses a wide variety of recombinant proteins. These proteins include, but are not limited to, glial-derived neurotrophic factor (GDNF), transforming growth factor-beta1 (TGF-beta1), TGF-beta2, TGF-beta3, inhibin A, inhibin B, bone morphogenetic protein-2 (BMP-2), BMP-4, inhibin alpha, Mullerian inhibiting substance (MIS), OP-1 (osteogenic protein 1), which are all members of the TGF-beta superfamily. The monomer subunits of the TGF-beta superfamily share certain structural features: they generally contain 8 highly conserved cysteine residues that form 4 intramolecular disulfides. Typically a ninth conserved cysteine is free in the monomeric form of the protein but participates in an intermolecular disulfide bond formed during the homodimerization or heterodimerization of the monomer subunits. Other members of the TGF-beta superfamily are described by Massague (1990), Daopin et al. (1992), Kingsley (1994), Kutty et al. (1998), and Lawton et al. (1997), incorporated herein by reference.

Immunoglobulin heavy and light chain monomers also contain cysteine residues that participate in intramolecular disulfides as well as free cysteines (Roitt et al., 1989 and Paul, 1989). These free cysteines normally only participate in disulfide bonds as a consequence of multimerization events such as heavy chain homodimerization, heavy chain – light chain heterodimerization, homodimerization of the (heavy chain – light chain) heterodimers, and other higher order assemblies such as pentamerization of the (heavy chain – light chain) heterodimers in the case of IgM. Thus, the methods of the present invention can be employed to enhance the expression, recovery and purification of heavy and/or light chains (or various domains thereof) of human immunoglobulins such as IgG1, IgG2, IgG3, IgG4, IgM IgA1, IgA2, secretory IgA, IgD and IgE. Immunoglobulins from other species could also be similarly expressed, recovered and purified. Proteins genetically fused to immunoglobulins or immunoglobulin domains as described in Chamow & Ashkenazi (1996) could also be similarly expressed, recovered and purified.

The present methods can also enhance the expression, recovery and purification of additional recombinant proteins including members of growth hormone superfamily. The following proteins are encoded by genes of the growth hormone (GH) supergene family (Bazan (1990); Bazan (1991); Mott and Campbell (1995); Silvennoinen and Ihle (1996); Martin et al., 1990; Hannum et al., 1994): growth hormone, prolactin, placental lactogen, erythropoietin (EPO), thrombopoietin (TPO), interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p35 subunit), IL-13, IL-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony

stimulating factor (M-CSF), cardiotrophin-1 (CT-1), Stem Cell Factor and the flt3/flk2 ligand ("the GH supergene family"). It is anticipated that additional members of this gene family will be identified in the future through gene cloning and sequencing. Members of the GH supergene family have similar secondary and tertiary structures, despite the fact that they generally have limited amino acid or DNA sequence identity. The shared structural features allow new members of the gene family to be readily identified.

A group of proteins has been classed as a structural superfamily based on the shared structural motif termed the "cystine knot". The cystine knot is defined by six conserved cysteine residues that form three intramolecular disulfide bonds that are topologically "knotted" (McDonald and Hendrickson, 1993). These proteins also form homo- or heterodimers and in some but not all instances dimerization involves intermolecular disulfide formation. Members of this family include the members of the TGF-beta superfamily and other proteins such as platelet derived growth factor-A (PDGF-A), PDGF-B, nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4, and vascular endothelial growth factor (VEGF). Cystine and other cysteine blocking reagents could also enhance expression, recovery and purification of proteins with this structural motif.

The present methods can also enhance the expression, recovery and purification of other recombinant proteins and/or cysteine added variants of those proteins. Classes of proteins would include proteases and other enzymes, protease inhibitors, cytokines, cytokine antagonists, allergens, chemokines, gonadotrophins, chemotactins, lipid-binding proteins, pituitary hormones, growth factors, somatomedans, immunoglobulins, interleukins, interferons, soluble receptors, vaccines, and hemoglobins. Specific examples of proteins include, for example, leptin, insulin, insulin-like growth factor 1 (IGF1), superoxide dismutase, catalase, asparaginase, uricase, fibroblast growth factors, arginase, phenylalanine ammonia, angiostatin, endostatin. Factor VIII, Factor IX, interleukin 1 receptor antagonist, protease nexin and anti-thrombin III.

Other protein variants that would benefit from PEGylation and would therefore be reasonable candidates for cysteine added modifications include proteins or peptides with poor solubility or a tendency to aggregate, proteins or peptides that are susceptible to proteolysis, proteins or peptides needing improved mechanical stability, proteins or peptides that are cleared rapidly from the body, or proteins or peptides with undesirable immunogenic or antigenic properties.

If desired, muteins of natural proteins can be generally constructed using site-directed PCR-based mutagenesis as described in general in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications edited by White, B. A. (1993) Humana Press, Inc., Totowa, NJ and PCR Protocols: A Guide to Methods and Applications edited by Innis, M. A. et al. (1990) Academic Press, Inc. San Diego, CA. Typically, PCR primer oligonucleotides are designed to incorporate nucleotide changes to the coding sequence of proteins that result in substitution of a cysteine residue for an amino acid at a specific position within the protein. Such mutagenic oligonucleotide primers can also be designed to incorporate an additional cysteine residue at the carboxy terminus or amino terminus of the coding sequence of proteins. In this latter case one or more additional amino acid

residues could also be incorporated amino terminal and/or carboxy terminal to the added cysteine residue if that were desirable. Moreover oligonucleotides can be designed to incorporate cysteine residues as insertion mutations at specific positions within the protein coding sequence if that were desirable. Again, one or more additional amino acids could be inserted along with the cysteine residue and these amino acids could be positioned amino terminal and/or carboxy terminal to the cysteine residue.

The choice of sequences for mutagenic oligos is dictated by the position where the desired cysteine residue is to be placed and the propinquity of useful restriction endonuclease sites. Generally it is desirable to place the mutation, i.e. the mis-matched segment near the middle of the oligo to enhance the annealing of the oligo to the template. It is also desirable for the mutagenic oligo to span a unique restriction site so that the PCR product can be cleaved to generate a fragment that can be readily cloned into a suitable vector. An example would be one that can be used to express the mutein or that provides convenient restriction sites for excising the mutated gene and readily cloning it into such an expression vector. It is generally desirable to employ mutagenic oligos under 80 bases in length and lengths of 30-40 bases are more preferable. Sometimes mutation sites and restriction sites are separated by distances that are greater than that which is desirable for synthesis of synthetic oligonucleotides. In such instances, multiple rounds of PCR can be employed to incrementally extend the length of the PCR product such that it includes the desired useful restriction site or genes targeted for mutagenesis can be reengineered or resynthesized to incorporate restriction sites at appropriate positions. Alternatively, variations of PCR mutagenesis protocols, such as the so-called "Megaprimer Method" (Barik, S. pp277-286 in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications edited by White, B. A. (1993) Humana Press, Inc., Totowa, NJ) or "Gene Splicing by Overlap Extension" (Horton, R. M. pp251-261 in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications edited by White, B. A. (1993) Humana Press, Inc., Totowa, NJ), both incorporated herein by reference, can also be employed to construct such mutations.

Next, the host cell is exposed to a cysteine blocking agent. In one embodiment, the blocking agent is present at the time of cell disruption, and preferably is added prior to disrupting the cells. Cell disruption can be accomplished by, for example, mechanical sheer such as a French pressure cell, enzymatic digestion, sonication, homogenization, glass bead vortexing, detergent treatment, organic solvents, freeze thaw, grinding with alumina or sand and the like (Bollag et al., 1996).

In an alternative embodiment, the cysteine blocking agent can be exposed to the host cell before, during or after the host cell is induced to express the desired protein. For example, the host cell can be cultured in the presence of the cysteine blocking agent, which might be preferred for expression of proteins that are secreted into the media such as erythropoietin, for example. Alternatively, prior to or at the time of, exposing the host cell to the cysteine blocking agent, the host cell can be induced to express the desired protein, either as a secreted protein in the periplasm or media, or as a cytoplasmic protein. Methods known in the art can be used to induce such expression in the cytoplasm or to direct secretion depending on cell origin, including, for example, the methods

described in the examples below. A wide variety of signal peptides have been used successfully to transport proteins to the periplasmic space. Examples of these include prokaryotic signal sequences such as ompA, stII, PhoA signal (Denefle et al., 1989), OmpT (Johnson et al., 1996), LamB and OmpF (Hoffman and Wright, 1985), beta-lactamase (Kadonaga et al., 1984), enterotoxins LT-A, LT-B (Morioka-Fujimoto et al., 1991), and protein A from *S. aureus* (Abrahmsen et al., 1986). A number of non-natural, synthetic, signal sequences that facilitate secretion of certain proteins are also known to those skilled in the art.

For proteins secreted to the periplasm, an osmotic shock treatment can be used to selectively disrupt the outer membrane of the host cells with the resulting release of periplasmic proteins. The osmotic shock buffer can be any known in the art, including, for example, the osmotic shock buffer and procedures described in Hsiung et al. (1986), or as described in the examples below. For proteins secreted into the media, preferably the media should contain the cysteine blocking agent during the time the cells express and secrete the protein. The cysteine blocking agent also could be added to the media following secretion of the protein but prior to purification of the protein.

The cysteine blocking agent can be added to the culture media at a concentration in the range of about 0.1 μ M to about 100 mM. Preferably, the concentration of cysteine blocking agent in the media is about 50 μ M to about 5 mM

Although not wishing to be bound by any particular theory, it is believed that the cysteine blocking agents used in the present methods covalently attach to the "free" cysteine residue, forming a mixed disulfide, thus stabilizing the free cysteine residue and preventing multimerization and aggregation of the protein. Alternatively, the presence of an oxidizing agent in the osmotic shock buffers may be augmenting the protein refold process in case incomplete renaturation had occurred following secretion of the protein into the periplasmic space. However, as noted above, periplasmic protein refolding can be inefficient. For this reason it is believed that addition of cystine to osmotic shock buffers also may increase the recovery of recombinant proteins containing an even number of cysteine residues, even if the cysteines normally form disulfide bonds. In addition, the inventors believe it may be advantageous to add cystine or similar compounds to the fermentation media during bacterial growth because these compounds should diffuse into the periplasm due to the porous nature of the bacterial outer membrane. Protein folding and blocking of the free cysteine can be accomplished before cell recovery and lysis. Early protein stabilization protects against proteolysis and can contribute to higher recovery yields of recombinant proteins.

A number of thiol-reactive compounds can be used as cysteine blocking agents to stabilize proteins containing free cysteines. In addition to cystine, blocking agents can also include reagents containing disulfide linkages such as cystamine, dithioglycolic acid, oxidized glutathione, 5,5'-dithiobis(2-nitrobenzoic acid (Ellman's reagent), pyridine disulfides, compounds of the type R-S-S-CO-OCH₃, other derivatives of cystine such as diformylcystine, diacetylcystine, diglycylcystine, dialanylcystine diglutaminylcystine, cystinyldiglycine, cystinyldiglutamine, dialanylcystine dianhydride, cystine phenylhydantoin, homocystine, dithiodipropionic acid, dimethylcystine, or any dithiol or chemical capable of undergoing a disulfide exchange reaction. Sulfenyl halides can also be

used to prepare mixed disulfides. Other thiol blocking agents that may find use in stabilizing cysteine added protein variants include compounds that are able to reversibly react with free thiols. These agents include certain heavy metals salts or organic derivatives of zinc, mercury, and silver. Other mercaptide forming agents or reversible thiol reactive compounds are described by R. Cecil and J. R.

5 McPhee (1959) and Torchinskii (1971).

In the final step of the general method, the desired protein is recovered and purified from the soluble cytoplasmic fraction, the soluble periplasmic fraction or the soluble fraction of the media. Any method for recovering and purifying proteins from the media, cytoplasmic or periplasmic fraction can be used. Such recovery and purification methods are known or readily determined by those skilled in the art, including, for example, centrifugation, filtration, dialysis, chromatography, including size exclusion, procedures and the like. A suitable method for the recovery and purification of a desired protein will depend, in part, on the properties of the protein and the intended use.

The present invention further provides novel methods for producing soluble interferon, particularly alpha interferon, that results in a significant increase in the percent of the recovered interferon that has been properly processed. These methods include culturing host cells capable of expressing interferon at a pH range of about 5 to about 6.5, and preferably about 5.5 to about 6.5. Published reports (Voss et al. (1994)) using a higher pH only resulted in 50% properly processed interferon, whereas the new methods of the present invention at lower pHs recovered about 80-90%.

20 A discovery was made that certain of the monomeric hGH cysteine variants formed disulfide-linked dimeric hGH proteins during the chromatography procedures used to purify these proteins. The disulfide-linked hGH dimers formed when cystine was removed from the column buffers. New procedures were developed to purify the disulfide-linked dimeric hGH proteins because the disulfide-linked dimeric hGH proteins behaved differently than monomeric hGH proteins during the column chromatography steps used to purify the proteins. Unexpectedly, it was discovered that the disulfide-linked dimeric hGH proteins were biologically active in *in vitro* bioassays. Biologically active, homogeneous, disulfide-linked dimeric hGH proteins are novel. Accordingly, the present invention further relates to these biologically active, homogeneous di-sulfide lined, dimeric hGH proteins as well. Higher order multimers are also contemplated, including trimers, tetramers and the like, as described in the examples below.

The purified proteins can then be further processed if desired. For example, the proteins can be PEGylated at the free cysteine site with various cysteine-reactive PEG reagents, and subsequently purified as monoPEGylated proteins. The term "monoPEGylated" is defined to mean a protein modified by covalent attachment of a single PEG molecule at a specific site on the protein. Any method known to those skilled in the art can be used, including, for example, the methods described in the examples below, particularly Example 11.

Braxton (1998) teaches methods for PEGylating cysteine muteins of proteins, and in particular cysteine muteins of GH and erythropoietin. Braxton (1998) specifically teaches that the buffers used to PEGylate the cysteine muteins should not contain a reducing agent. Examples of

reducing agents provided by Braxton (1998) are beta-mercaptoethanol (BME) and dithiothreitol (DTT). When similar procedures were used to PEGylate cysteine muteins of GH, erythropoietin and alpha interferon, it was discovered that the cysteine muteins did not PEGylate. It has now been discovered that treatment of the purified cysteine muteins with a reducing agent is required for the proteins to be PEGylated. Although not wanting to be bound by any particular theory, the inventors believe that the reducing agent is required to reduce the mixed disulfide and expose the free cysteine residue in the protein so that the free cysteine can react with the PEG reagent. Thus, the present invention also relates to methods for PEGylating cysteine muteins of GH, erythropoietin, alpha interferon and other proteins containing $2N + 1$ cysteine residues, proteins containing $2N$ cysteine residues where two or more of the cysteine residues are free, particularly those muteins and proteins in which the free cysteine residue is blocked by a mixed disulfide.

The present invention further relates to purified, monoPEGylated protein variants produced by the methods disclosed herein that are not only biologically active, but also retain high specific activity in protein-dependent mammalian cell proliferation assays. Such protein variants include, for example, the following purified, monoPEGylated cysteine muteins: hGH, EPO and alpha IFN. For example, the *in vitro* biological activities of the monoPEGylated hGH variants were 10- to 100-fold greater than the biological activity of hGH that has been PEGylated using NHS-PEG reagents.

In one embodiment of the monoPEGylated hGH, the polyethylene glycol is attached to the C-D loop of hGH and the resulting monoPEGylated hGH has an EC_{50} less than about 110 ng/ml (5 nM), preferably less than about 50 ng/ml (2.3 nM). Alternatively, the polyethylene glycol moiety can be attached to a region proximal to the Helix A of hGH and the resulting monoPEGylated hGH has an EC_{50} less than about 110 ng/ml (5nM), preferably less than 11 ng/ml (0.5nM), and more preferably less than about 2.2 ng/ml (0.1 nM).

In one embodiment of the monoPEGylated EPO, the polyethylene glycol is attached to the C-D loop of EPO and the resulting monoPEGylated EPO has an EC_{50} less than about 1000 ng/ml (21 nM), preferably less than about 100 ng/ml (approximately 6 nM), more preferably less than about 10 ng/ml (approximately 0.6 nM) and most preferably less than about 1 ng/ml (approximately 0.06 nM). Alternatively, the polyethylene glycol moiety can be attached to the A-B loop of EPO and the resulting monoPEGylated EPO has an EC_{50} less than about 100 ng/ml (approximately 5nM), preferably less than 20 ng/ml (approximately 1 nM), and more preferably less than about 1 ng/ml (approximately 0.05 nM).

In one embodiment of the monoPEGylated alpha IFN, the polyethylene glycol is attached to the region proximal to Helix A of alpha IFN and the resulting monoPEGylated alpha IFN has an IC_{50} less than about 100 pg/ml (approximately 5 pM), more preferably less than about 50 pg/ml (approximately 2.5 pM) and most preferably about 22 ng/ml (approximately 1.2 pM). Alternatively, the polyethylene glycol moiety can be attached to the C-D loop of IFN- $\alpha 2$ and the resulting monoPEGylated IFN- $\alpha 2$ has an EC_{50} less than about 100 pg/ml (approximately 5 pM).

There are over 25 distinct IFN- α genes (Pestka et al., 1987). Members of the IFN- α family share varying degrees of amino acid homology and exhibit overlapping sets of biological activities.

Non-natural recombinant IFN- α s, created through joining together regions of different IFN- α proteins are in various stages of clinical development (Horisberger and DiMarco, 1995). A non-natural "consensus" interferon (Blatt et al., 1996), which incorporates the most common amino acid at each position of IFN- α , also has been described. Appropriate sites for PEGylating cysteine mutants of IFN- α 2 should be directly applicable to other members of the IFN- α gene family and to non-natural IFN- α s. Kinstler et al., (1996) described monoPEGylated consensus interferon in which the protein is preferentially mono PEGylated at the N-terminal, non-natural methionine residue. Bioactivity of the PEGylated protein was reduced approximately 5-fold relative to non-modified consensus interferon (Kinstler et al., 1996).

The present invention further provides protein variants that can be covalently attached or conjugated to each other or to a chemical group to produce higher order multimers, such as dimers, trimers and tetramers. Such higher order multimers can be produced according to methods known to those skilled in the art or as described in Example 15 below. For example, such a conjugation can produce a hGH, EPO or alpha IFN adduct having a greater molecular weight than the corresponding native protein. Chemical groups suitable for coupling are preferably non-toxic and non-immunogenic. These chemical groups would include carbohydrates or polymers such as polyols.

The "PEG moiety" useful for attaching to the cysteine variants of the present invention to form "pegylated" proteins include any suitable polymer, for example, a linear or branched chained polyol. A preferred polyol is polyethylene glycol, which is a synthetic polymer composed of ethylene oxide units. The ethylene oxide units can vary such that PEGylated-protein variants can be obtained with apparent molecular weights by size-exclusion chromatography ranging from approximately 30-500,000. The size of the PEG moiety directly impacts its circulating half-life Yamaoka et al. (1994). Accordingly, one could engineer protein variants with differing circulating half-lives for specific therapeutic applications or preferred dosing regimes by varying the size or structure of the PEG moiety. Thus, the present invention encompasses GH protein variants having an apparent molecular weight greater than about 30 kDa, and more preferably greater than about 70 kDa as determined by size exclusion chromatography, with an EC_{50} less than about 400 ng/ml (18 nM), preferably less than 10 ng/ml (5 nM), more preferably less than about 10 ng/ml (0.5 nM), and even more preferably less than about 2.2 ng/ml (0.1 nM). The present invention further encompasses EPO protein variants having an apparent molecular weight greater than about 30 kDa, and more preferably greater than about 70 kDa as determined by size exclusion chromatography, with an EC_{50} less than about 1000 ng/ml (21 nM), preferably less than 100 ng/ml (6 nM), more preferably less than about 10 ng/ml (0.6 nM), and even more preferably less than about 1 ng/ml (0.06 nM). The present invention further encompasses alpha IFN (IFN- α) protein variants having an apparent molecular weight greater than about 30 kDa, and more preferably greater than about 70 kDa as determined by size exclusion chromatography, with an IC_{50} less than about 1900 pg/ml (100 pM), preferably less than 400 pg/ml (21 pM), more preferably less than 100 pg/ml (5 nM), and even more preferably less than about 38 pg/ml (2 pM).

The reactive PEG end group for cysteine modification includes but is not limited to vinylsulfone, maleimide and iodoacetyl moieties. The PEG end group should be specific for free thiols with the reaction occurring under conditions that are not detrimental to the protein.

Antagonist hGH variants also can be prepared using a cysteine-added variant GH where chemical derivatization does not interfere with receptor binding but does prohibit the signaling process. Conditions that would benefit from the administration of a GH antagonist include acromegaly, vascular eye diseases, diabetic nephropathy, restenosis following angioplasty and growth hormone responsive malignancies.

As used herein, the term "derivative" refers to any variant of a protein expressed and recovered by the present methods. Such variants include, but are not limited to, PEGylated versions, dimers and other higher order variants, amino acid variants, fusion proteins, changes in carbohydrate, phosphorylation or other attached groups found on natural proteins, and any other variants disclosed herein.

The compounds produced by the present methods can be used for a variety of *in vitro* and *in vivo* uses. The proteins and their derivatives of the present invention can be used for research, diagnostic or therapeutic purposes that are known for their wildtype, natural, or previously-known modified counterparts. *In vitro* uses include, for example, the use of the protein for screening, detecting and/or purifying other proteins.

For therapeutic uses, one skilled in the art can readily determine the appropriate dose, frequency of dosing and route of administration. Factors in making such determinations include, without limitation, the nature of the protein to be administered, the condition to be treated, potential patient compliance, the age and weight of the patient, and the like. The compounds of the present invention can also be used as delivery vehicles for enhancement of the circulating half-life of the therapeutics that are attached or for directing delivery to a specific target within the body.

The following examples are not intended to be limiting, but only exemplary of specific embodiments of the invention.

Example 1

Development of an *in vitro* Bioassay for Human Growth Hormone

An hGH cell proliferation assay that uses the murine FDC-P1 cell line stably transfected with the rabbit GH receptor was developed (Rowlinson et al., 1995). The mouse FDC-P1 cell line was purchased from the American Type Culture Collection and routinely propagated in RPMI 1640 media supplemented with 10% fetal calf serum, 50 µg/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine and 17-170 Units/ml mouse IL-3 (FDC-P1 media):

35

A. Cloning a cDNA Encoding the Rabbit GH Receptor

The rabbit GH receptor was cloned by PCR using forward primer BB3 (5'-CCCCGGATCCGCCACCATGGATCTCTGG CAGCTGCTGTT-3') (SEQ.ID.NO. 1) and reverse primer BB36 (5'-CCCCGTCGACTCTAGAGCCATTA GATACAAAGCTCT TGGG-3')

(SEQ.ID.NO. 2). BB3 anneals to the DNA sequence encoding the initiator methionine and amino terminal portion of the receptor. BB3 contains an optimized KOZAK sequence preceding the initiator methionine and a *Bam* HI site for cloning purposes. BB36 anneals to the 3' untranslated region of the rabbit GH receptor mRNA and contains *Xba* I and *Sal* I restriction sites for cloning purposes. Rabbit liver poly(A)⁺ mRNA (purchased from CLONTECH, Inc.) was used as the substrate in first strand synthesis of single-stranded cDNA to produce template for PCR amplification. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp. Parallel first strand cDNA syntheses were performed using random hexamers or BB36 as the primer. Subsequent PCR reactions using the products of the first strand syntheses as templates were carried out with primers BB3 and BB36. The expected ~ 1.9 kb PCR product was observed in PCR reactions using random hexamer-primed or BB36-primed cDNA as template. The random hexamer-primed cDNA was digested with *Bam* HI and *Xba* I, which generates two fragments (~ 365 bp and ~ 1600 bp) because the rabbit GH receptor gene contains an internal *Bam* HI site. Both fragments were gel-purified. The full-length rabbit GH receptor cDNA was then cloned in two steps. First the ~1600 bp *Bam* HI - *Xba* I fragment was cloned into pCDNA3.1(+) (Invitrogen Corporation) that had been digested with these same two enzymes. These clones were readily obtained at reasonable frequencies and showed no evidence of deletions as determined by restriction digests and subsequent sequencing. To complete the rabbit receptor cDNA clone, one of the sequenced plasmids containing the 1600 bp *Bam* HI - *Xba* I fragment was digested with *Bam* HI, treated with calf alkaline phosphatase, gel-purified and ligated with the gel-purified ~365 bp *Bam* HI fragment that contains the 5' portion of the rabbit GH receptor gene. Transformants from this ligation were picked and analyzed by restriction digestion and PCR to confirm the presence of the ~365 bp fragment and to determine its orientation relative to the distal segment of the rabbit GH receptor gene. The sequence for one full length clone was then verified. This plasmid, designated pBBT118, was used to stably transfect FDC-P1 cells.

B. Selection of Stably Transfected FDC-P1 Cells Expressing the Rabbit GH Receptor

Endotoxin-free pBBT118 DNA was prepared using a Qiagen "Endo-Free Plasmid Purification Kit" and used to transfect FDC-P1 cells. Mouse IL-3 was purchased from R&D Systems. FDC-P1 cells were transfected with plasmid pBBT118 using DMRIE-C cationized lipid reagent purchased from GIBCO, following the manufacturer's recommended directions. Briefly, 4 µg of plasmid DNA were complexed with 4-30 µl of the DMRIE-C reagent in 1 ml of OptiMEM media (GIBCO) for 45 minutes in six well tissue culture dishes. Following complex formation, 2x 10⁶ FDC-P1 cells in 200 µl of OptiMEM media supplemented with mouse IL-3 were added to each well and the mixture left for 4 h at 37°C. The final mouse IL-3 concentration was 17 Units/ml. Two ml of FDC-P1 media containing 15% fetal bovine serum were added to each well and the cells left overnight at 37°C. The next day transfected cells were transferred to T-75 tissue culture flasks containing 15 ml FDC-P1 media supplemented with IL-3 (17 U/ml), hGH (5 nM) and 10% horse serum rather than fetal calf serum. Horse serum was used because of reports that fetal calf serum contains a growth-promoting

activity for FDC-P1 cells. Three days later the cells were centrifuged and resuspended in fresh FDC-P1 media containing 400 µg/ml G418, 17 U/ml IL-3, 5 nM hGH, 10 % horse serum and incubated at 37°C. Media was changed every few days. The cells from each transfection were split into T-75 tissue culture flasks containing fresh media and either mouse IL-3 (17 U/ml) or hGH (5 nM). G418 resistant cells were obtained from both the IL-3- and hGH-containing flasks. The transformants used in the bioassays originated from flasks containing hGH. Twelve independent cell lines were selected by limiting dilution. Five of the cell lines (GH-R3, -R4, -R5, -R6 and -R9) showed a good proliferative response to hGH. Preliminary experiments indicated that the EC₅₀ for hGH was similar for each cell line, although the magnitude of the growth response varied depending upon the line. The GH-R4 cell line was studied in most detail and was used for the assays presented below. The cell lines were routinely propagated in RPMI 1640 media containing 10% horse serum, 50 Units/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, 400 µg/ml G418 and 2-5 nM pituitary hGH or rhGH.

15 C. Development of an hGH Bioassay Using FDC-P1 Expressing the Rabbit GH Receptor

A modified version of the MTT cell proliferation assay described by Rowlinson et al. (1995) was developed to measure hGH bioactivity. Our assay measures uptake and reduction of the dye MTS, which creates a soluble product, rather than MTT, which creates an insoluble product that must be solubilized with organic solvents. The advantage of using MTS is that absorbance of the wells can be determined without the need to lyse the cells with organic solvents.

GH-R4 cells were washed three times with phenol red-free RPMI 1640 media and suspended in assay media (phenol red-free RPMI 1640, 10% horse serum, 50 Units/ml penicillin, 50 µg/ml streptomycin, 400 µg/ml G418) at a concentration of 1×10^5 cells/ml. Fifty microliters of the cell suspension (5×10^3 cells) were added to wells of a 96 well flat bottomed tissue culture plate. Serial three-fold dilutions of protein samples were prepared in assay media and added to microtiter wells in a volume of 50 µl, yielding a final volume of 100 µl per well. Protein samples were assayed in triplicate wells. Plates were incubated at 37°C in a humidified 5% CO₂ tissue culture incubator for 66-72 h, at which time 20 µl of an MTS/PES (PES is an electron coupler) reagent mixture (CellTiter 96 Aqueous One solution reagent, Promega Corporation) was added to each well. Absorbance of the wells at 490 nm was measured 2-4 h later. Absorbance value means +/- standard deviations for the triplicate wells were calculated. Control wells contained media but no cells. Absorbance values for the control wells (typically 0.06 – 0.2 absorbance units) were subtracted from the absorbance values for the sample wells. Control experiments demonstrated that absorbance signals correlated with cell number up to absorbance values of 2. All assays included a human pituitary GH standard. Experiments utilizing the parental FDC-P1 cell line were performed as described above except that the assay media did not contain G418 and fetal calf serum was substituted for horse serum.

Example 2**Cloning and Expression of rhGH****A. Cloning a cDNA encoding human Growth Hormone (GH)**

A human GH cDNA was amplified from human pituitary single-stranded cDNA (commercially available from CLONTECH, Inc., Palo Alto, CA) using the polymerase chain reaction (PCR) technique and primers BB1 and BB2. The sequence of BB1 is (5'-GGGGGTCGACCATATGTTCCCAACCATTCCTTATCCAG-3')(SEQ.ID.NO. 3). The sequence of BB2 is (5'-GGGGGATCCTCACTAGAAGCCACAGCTGCCCTC-3')(SEQ.ID.NO. 4). Primer BB1 was designed to encode an initiator methionine preceding the first amino acid of mature GH, phenylalalanine, and *Sal* I and *Nde* I sites for cloning purposes. The reverse primer, BB2, contains a *Bam* HI site for cloning purposes. The PCR reactions contained 20 pmoles of each oligo primer, 1X PCR buffer (Perkin-Elmer buffer containing MgCl₂), 200 μM concentration of each of the four nucleotides dA, dC, dG and dT, 2 ng of single-stranded cDNA, 2.5 units of *Taq* polymerase (Perkin-Elmer) and 2.5 units of Pfu polymerase (Stratagene, Inc). The PCR reaction conditions were: 96°C for 3 minutes, 35 cycles of (95°C, 1 minute; 63°C for 30 seconds; 72°C for 1 minute), followed by 10 minutes at 72°C. The thermocycler employed was the Ampliion II Thermal Cycler (Thermolyne) The approximate 600 bp PCR product was digested with *Sal* I and *Bam* HI, gel-purified and cloned into similarly digested plasmid pUC19 (commercially available from New England BioLabs, Beverly, MA). The ligation mixture was transformed into *E. coli* strain DH5alpha and transformants selected on LB plates containing ampicillin. Several colonies were grown overnight in LB media and plasmid DNA isolated using miniplasmid DNA isolation kits purchased from Qiagen, Inc (Valencia, CA). Clone LB6 was determined to have the correct DNA sequence.

For expression in *E. coli*, clone LB6 was digested with *Nde* I and *Eco* RI, the approximate 600 bp fragment gel-purified, and cloned into plasmid pCYB1 (commercially available from New England Biolabs, Beverly, MA) that had been digested with the same enzymes and treated with calf alkaline phosphatase. The ligation mixture was transformed into *E. coli* DH5alpha and transformants selected on LB ampicillin plates. Plasmid DNA was isolated from several transformants and screened by digestion with *Nde* I and *Eco* RI. A correct clone was identified and named pCYB1: wtGH (pBBT120). This plasmid was transformed into *E. coli* strains JM109 or W3110 (available from New England BioLabs and the American Type Culture Collection).

B. Construction of stII-GH

Wild type GH clone LB6 (pUC19: wild type GH) was used as the template to construct a GH clone containing the *E. coli* stII signal sequence. Because of its length, the stII sequence was added in two sequential PCR reactions. The first reaction used forward primer BB12 and reverse primer BB10. BB10 has the sequence (5'-CGCGGATCCGATTAGAATCCACAGCTCCCCTC 3')(SEQ.ID.NO. 5). BB12 has the sequence (5'-GCATCTATGTTCTGTTTTCTCTATCGCTACCAACGCTTACGCA TTCCCAACCATTCCTTATCCAG-3')(SEQ.ID.NO. 6). The PCR reactions were as described for amplifying wild type GH. The approximate 630 bp PCR product was gel-purified using the Qiaex II

Gel Extraction Kit (Qiagen, Inc), diluted 50-fold in water and 2 µl used as template for the second PCR reaction. The second PCR reaction used reverse primer BB10 and forward primer BB11. BB11 has the sequence (5'CCCCCTCTAGACATATGAAGAAGAACATCGCATTCTGCTGGCATCTATGTTTCGTTTTCTCTATCG-3')(SEQ.ID.NO. 7). Primer BB11 contains *Xba*I and *Nde*I sites for cloning purposes. PCR conditions were as described for the first reaction. The approximate 660 bp PCR product was digested with *Xba*I and *Bam*HI, gel-purified and cloned into similarly cut plasmid pCDNA3.1(+) (Invitrogen, Inc. Carlsbad, CA). Clone pCDNA3.1(+):stII-GH(5C) or "5C" was determined to have the correct DNA sequence.

Clone "5C" was cleaved with *Nde*I and *Bam*HI and cloned into similarly cut pBBT108 (a derivative of pUC19 which lacks a *Pst* I site, this plasmid is described below). A clone with the correct insert was identified following digestion with these enzymes. This clone, designated pBBT111, was digested with *Nde* I and *Sal* I, the 660 bp fragment containing the stII-GH fusion gene, was gel-purified and cloned into the plasmid expression vector pCYB1 (New England BioLabs) that had been digested with the same enzymes and treated with calf alkaline phosphatase. A recombinant plasmid containing the stII-GH insertion was identified by restriction endonuclease digestions. One such isolate was chosen for further studies and was designated pBBT114. This plasmid was transformed into *E. coli* strains JM109 or W3110 (available from New England BioLabs and the American Type Culture Collection).

20 C. Construction of ompA-GH

Wild type GH clone LB6 (pUC19: wild type GH) was used as the template to construct a GH clone containing the *E. coli* ompA signal sequence. Because of its length, the ompA sequence was added in two sequential PCR reactions. The first reaction used forward primer BB7 (5'GCAGTGGC ACTGGCTGGTTTCGCTACCGTAGCGCAGGCCTCCCAACCATTCCTTATCCAG 3')(SEQ.ID.NO. 8) and reverse primer BB10: (5' CGCGGATCCGATTAGAATCC ACAGCTCCCCCTC 3')(SEQ.ID.NO. 5). The PCR reactions were as described for amplifying wild type GH except that approximately 4 ng of plasmid LB6 was used as the template rather than single-stranded cDNA and the PCR conditions were 96°C for 3 minutes, 30 cycles of (95°C for 1 minute; 63°C for 30 seconds; 72°C for 1 minute) followed by 72°C for 10 minutes. The approximate 630 bp PCR product was gel-purified using the Qiaex II Gel Extraction Kit (Qiagen, Inc), diluted 50-fold in water and 2 µl used as template for the second PCR reaction. The second PCR reaction used reverse primer BB10 and forward primer BB6: (5'CCCCG TCGACACATATGAAGAAGACAGCTATCGCGATTGCAGT GGCCTGGCTGGTTTC 3')(SEQ.ID.NO. 9). PCR conditions were as described for the first reaction. The approximate 660 bp PCR product was gel-purified, digested with *Sal* I and *Bam* HI and cloned into pUC19 (New England BioLabs) which was cut with *Sal* I and *Bam* HI or pCDNA3.1(+) (Invitrogen) which had been cut by *Xho* I and *Bam* HI (*Sal* I and *Xho* I produce compatible single-stranded overhangs). When several clones were sequenced, it was discovered that all pUC19 (8/8) clones contained errors in the region of the ompA sequence. Only one pCDNA3.1(+) clone was sequenced and it contained a

sequence ambiguity in the ompA region. In order to generate a correct ompA-GH fusion, gene segments of two sequenced clones, which contained different errors separated by a convenient restriction site were recombined and cloned into the pUC19-derivative that lacks the *Pst* I site (see pBBT108 described below). The resulting plasmid, termed pBBT112, carries the ompA-GH fusion gene cloned as an *Nde* I - *Bam* HI fragment into these same sites in pBBT108. This plasmid is designated pBBT112 and is used in PCR-based, site-specific mutagenesis of GH as described below.

D. Construction of *Pst* I pUC19 (pBBT 108)

To facilitate mutagenesis of the cloned GH gene for construction of selected cysteine substitution and insertion mutations, a derivative of the plasmid pUC19 (New England BioLabs) lacking a *Pst* I site was constructed as follows. pUC19 plasmid DNA was digested with *Pst* I and subsequently treated at 75°C with Pfu DNA Polymerase (Stratagene) using the vendor-supplied reaction buffer supplemented with 200 µM dNTPs. Under these conditions the polymerase will digest the 3' single-stranded overhang created by *Pst* I digestion but will not digest into the double-stranded region and the net result will be the deletion of the 4 single-stranded bases which comprise the middle four bases of the *Pst* I recognition site. The resulting molecule has double-stranded, i.e. "blunt", ends. Following these enzymatic reactions the linear monomer was gel-purified using the Qiaex II Gel Extraction Kit (Qiagen, Inc). This purified DNA was treated with T4 DNA Ligase (New England BioLabs) according to the vendor protocols, digested with *Pst* I, and used to transform *E. coli* DH5alpha. Transformants were picked and analyzed by restriction digestion with *Pst* I and *Bam* HI. One of the transformants which was not cleaved by *Pst* I but was cleaved at the nearby *Bam* HI site was picked and designated pBBT108.

E. Expression of met-hGH and stII-hGH in *E. coli*

pBBT120, which encodes met-hGH, and pBBT114, which encodes stII-hGH were transformed into *E. coli* strain W3110. The parental vector pCYB1 also was transformed into W3110. The resulting strains were given the following designations:

BOB130: W3110 (pCYB1) = vector only

BOB133: W3110 (pBBT120) = met-hGH

BOB132: W3110 (pBBT114) = stII-hGH

These strains were grown overnight at 37°C in Luria Broth (LB) containing 100 µg/ml ampicillin. The saturated overnight cultures were diluted to ~0.025 O.D.s at A₆₀₀ in LB containing 100 µg/ml ampicillin and incubated at 37°C in shake flasks. When culture O.D.s reached ~0.25 - 0.5, IPTG was added to a final concentration of 0.5 mM to induce expression of the recombinant proteins. For initial experiments, cultures were sampled at 0, 1, 3, 5 and ~16 h post-induction. Samples of induced and uninduced cultures were pelleted and resuspended in SDS-PAGE sample buffer with the addition of 1% β- mercaptoethanol (BME) when desirable. Samples were electrophoresed on precast 14% Tris-glycine polyacrylamide gels. Gels were stained with Coomassie Blue or were analyzed by Western blotting.

Coomassie staining of whole cell lysates from strains BOB133, expressing met-hGH, and BOB132, expressing stII-hGH, showed a band of ~22 kDa that co-migrated with a purified rhGH standard purchased from Research Diagnostics Inc. The rhGH band was most prominent in induced cultures following overnight induction. Western blots using a polyclonal rabbit anti-hGH antiserum purchased from United States Biological, Inc. confirmed the presence of rhGH in lysates of induced cultures of BOB132 and BOB133 at both 3 and 16 h post-induction. No rhGH was detected by Western Blotting of induced cultures of the control strain BOB130 (vector only) at 3 or 16 h post-induction.

An induced culture of BOB132 (expressing stII-hGH) was prepared as described above and subjected to osmotic shock based on the analytical procedure of Koshland and Botstein (1980). This procedure ruptures the *E. coli* outer membrane and releases the contents of the periplasm into the surrounding medium. Subsequent centrifugation separates the soluble periplasmic components (recovered in the supernatant) from cytoplasmic, insoluble periplasmic, and cell-associated components (recovered in the pellet). Specifically, *E. coli* strain W3110 containing the st-II hGH plasmid was grown at 37°C overnight in LB containing 100 µg/ml ampicillin. The saturated overnight culture was diluted to 0.03 O.D. at A₆₀₀ in 25 ml of LB containing 100 µg/ml ampicillin and incubated at 37°C in a 250 ml shake flask. When the culture O.D. reached approximately 0.4, 100 mM IPTG was added for a final concentration of 0.5 mM to induce expression of the recombinant protein. The induced culture was then incubated at 37°C overnight (~16 h). The induced overnight culture reached an O.D. of 3.3 at A₆₀₀ and 4 O.D.s (1.2 ml) was centrifuged in a Eppendorf model 5415C microfuge at 14,000 rpm for 5 minutes at 4°C. The cell pellet was resuspended to approximately 10 O.D. in ice cold 20% sucrose, 10 mM Tris-HCl pH 8.0 by trituration and vortexing and EDTA pH 8.0 was added for a final concentration of 17 mM. Resuspended cells were incubated on ice for 10 minutes and centrifuged as above. The resultant pellet was resuspended at 10 O.D.s in ice cold water by trituration and vortexing and incubated on ice for 10 minutes. The resuspended cells were then centrifuged as above and the resultant supernatant (soluble periplasmic fraction) and cell pellet (insoluble periplasmic and cell associated components) were analyzed by SDS-PAGE.

The bulk of the rhGH synthesized by BOB132 was found to be soluble and localized to the periplasm. The periplasmic rhGH protein was indistinguishable in size from a purified pituitary hGH standard indicating that the stII signal sequence was removed during protein secretion.

Example 3

Purification and Bioactivity of rhGH

A. Purification of wild-type rhGH

In order to purify a significant quantity of wild-type rhGH, a 330 ml culture of BOB132 (expressing stII-hGH) was induced, cultured overnight and subjected to osmotic shock based on the preparative procedure described by Hsiung et al. (1986). Specifically, *E. coli* strain W3110 containing the st-II hGH plasmid was grown at 37°C overnight in LB containing 100 µg/ml ampicillin. The saturated overnight culture was diluted to 0.03 O.D. at A₆₀₀ in 2 X 250 ml (500 ml total volume) of

LB containing 100 µg/ml ampicillin and incubated at 37°C in 2 L shake flasks. When the culture's O.D. reached approximately 0.4, 100mM IPTG was added for a final concentration of 0.5 mM to induce expression of the recombinant protein. The induced culture was incubated at 37°C overnight (~16 h). The induced overnight cultures reached an O.D. of approximately 3.8 at A₆₀₀ and were

5 centrifuged using a Sorval RC-5 centrifuge and a GSA rotor at 8,000 rpm for 5 minutes at 4°C. The cell pellets were combined and resuspended to approximately 47 O.D. in ice cold 20% sucrose, 10 mM Tris-HCl pH 8.0 by trituration and EDTA pH 8.0 was added for a final concentration of ~25 mM. Resuspended cells were incubated on ice for 10 minutes and centrifuged in an IEC Centra MP4R centrifuge with an 854 rotor at 8,500 rpm for 7 minutes at 4°C. The resultant pellets were

10 resuspended at 47 O.D. in ice cold water by trituration and vortexing and incubated on ice for 30 minutes. The resuspended cells were centrifuged in the IEC centrifuge as above and the resultant supernatant (soluble periplasmic fraction) and cell pellet (insoluble periplasmic and cell associated components) were analyzed by SDS-PAGE. Again the gel showed that rhGH produced was soluble, periplasmic, and indistinguishable in size from the pituitary hGH standard.

15 rhGH was purified in a two step procedure based on that described by Becker and Hsiung (1986). The supernatant from the osmotic shock was loaded onto a 5 ml Pharmacia HiTrap Q Sepharose column equilibrated in 10 mM Tris-HCl pH 8.0 and the bound proteins were eluted with a 15 column volume 50-250 mM linear NaCl gradient. Column fractions were analyzed by SDS-PAGE. Fractions 22-25 eluting at a salt concentration of around 100-125 mM were enriched for hGH, and

20 were pooled, concentrated and further fractionated on a Superdex 200 HR 10/30 sizing column. Fractions 34-36 from the Superdex column (representing MWs around 21-22 kDa based on the elution profile of MW standards) contained most of the rhGH, were pooled and stored as frozen aliquots at -80°C. The final yield of rhGH, as determined by absorbance at 280 nm and by using a Bradford protein assay kit (Bio-Rad Laboratories), was about 2 mg. Non-reduced pituitary hGH

25 migrates with a slightly smaller apparent molecular weight than reduced pituitary hGH when analyzed by SDS-PAGE. This molecular weight change is indicative of proper disulfide bond formation. Our purified rhGH co-migrated with pituitary hGH under both reducing and non-reducing conditions indicating that the rhGH was properly folded and disulfide-bonded. Data presented below indicates that the biological activity of rhGH is indistinguishable from that of pituitary hGH.

30 **B. Bioassay results for Pituitary hGH and rhGH**

The parental FDC-P1 cell line shows a strong proliferative response to mouse IL-3, but not to pituitary hGH. In the absence of IL-3, the majority of FDC-P1 cells die, giving absorbance values less than 0.2. In contrast, FDC-P1 cells transformed with the rabbit growth hormone receptor proliferate in response to pituitary hGH, as evidenced by a dose-dependent increase in cell number

35 and absorbance values. The EC₅₀ (protein concentration required to achieve half-maximal stimulation) for this effect ranged from 0.75- 1.2 ng/ml pituitary hGH (0.03-0.05 nM) in different experiments, similar to what has been reported in the literature (Rowlinson et al., 1995). A significant difference between the parental FDC-P1 line and FDC-P1 cells transformed with the rabbit growth hormone receptor is that the latter cells survive in the absence of IL-3 or hGH, resulting in

higher absorbance values (typically 0.6 – 1.1, depending upon the assay and length of incubation with MTS in the zero growth factor control wells). The initial pool of rabbit growth hormone receptor transformants and all five independent growth hormone receptor cell lines isolated showed the same effect. A similar result was obtained with a second set of independently isolated rabbit growth hormone receptor transfectants. Rowlinson et al. (1995) observed a similar effect, suggesting that IL-3/GH-independent survival is a consequence of the transformation procedure. Although the growth hormone receptor cell lines did not require IL-3 or hGH for growth, they still showed a robust proliferative response to IL-3 and hGH. The practical effect of the higher absorbance values in the absence of hGH is to decrease the “window” of the hGH response (the difference between the maximum and minimum absorbance values). This window consistently ranged from 40 - 70% of the zero growth factor values, similar to what was reported by Rowlinson et al. (1995).

Pituitary GH and wild-type rhGH prepared by us had similar dose response curves in the bioassay with EC₅₀s ranging from 0.6-1.2 ng/ml in different experiments (Table 1).

15

Table 1
Bioactivities of hGH and hGH Cysteine Muteins

	Form Assayed	Mean EC₅₀ (ng/ml)	EC₅₀ Range (ng/ml)¹
Pituitary hGH	Monomer	1 +/- 0.1 (N=11)	0.75 – 1.2
RhGH	Monomer	0.8 +/- 0.3 (N=3)	0.6, 0.8, 1.1
T3C	Dimer	1.4 +/- 0.6 (N=7)	0.75 - 2.5
S144C	Monomer	1.6 +/- 0.8 (N=5)	1.1, 1.1, 1.5, 2.2, 2.7
T148C-lotA	Monomer	0.5 (N=2)	0.4, 0.5
T148C-lotB	Monomer	2.5 +/- 1.0 (N=4)	1.5, 1.9, 3.1, 3.6

N/A, not applicable

¹ EC₅₀ values from individual experiments. An EC₅₀ range is shown when N>5.

20

Example 4

Construction of hGH cysteine muteins

The cysteine substitution mutation T135C was constructed as follows. The mutagenic reverse oligonucleotide BB28 (5'-CTGCTTGAAGATCTGCCCACACCG GGGG CTGCCATC-3') (SEQ.ID.NO. 10) was designed to change the codon ACT for threonine at amino acid residue 135 to a TGT codon encoding cysteine and to span the nearby *Bgl* II site. This oligo was used in PCR along with BB34 (5'-GTAGCGCAGGCCCTTCCCAACC ATT-3') (SEQ.ID.NO. 11) which anneals to the junction region of the ompA-GH fusion gene and is not mutagenic. The PCR was performed in a 50 µl reaction in 1X PCR buffer (Perkin-Elmer buffer containing 1.5 mM MgCl₂), 200 µM concentration of each of the four nucleotides dA, dC, dG and dT, with each oligonucleotide primer present at 0.5 µM, 5 pg of pBBT112 (described above) as template and 1.25 units of AmpliTaq DNA Polymerase (Perkin-Elmer) and 0.125 units of Pfu DNA Polymerase (Stratagene). Reactions were performed in a Robocycler Gradient 96 thermal cycler (Stratagene). The program used entailed: 95°C for 3 minutes followed by 25 cycles of [95°C for 60 seconds, 45°C or 50°C or 55°C for 75 seconds, 72°C for 60 seconds] followed by a hold at 6° C. The PCR reactions were analyzed by

agarose gel electrophoresis which showed that all three different annealing temperatures gave significant product of the expected size; ~430 bp. The 45°C reaction was "cleaned up" using the QIAquick PCR Purification Kit (Qiagen) and digested with *Bgl* II and *Pst* I. The resulting 278 bp *Bgl* II-*Pst* I fragment, which includes the putative T135C mutation, was gel-purified and ligated into pBBT111, the pUC19 derivative carrying the stII-GH fusion gene (described above) which had been digested with *Bgl* II and *Pst* I and gel-purified. Transformants from this ligation were initially screened by digestion with *Bgl* II and *Pst* I and subsequently one clone was sequenced to confirm the presence of the T135C mutation and the absence of any additional mutations that could potentially be introduced by the PCR reaction or by the synthetic oligonucleotides. The sequenced clone was found to have the correct sequence throughout the *Bgl* II – *Pst* I segment.

The substitution mutation S132C was constructed using the protocol described above for T135C with the following differences: mutagenic reverse oligonucleotide BB29 (5>CTGCTTGAA GATCTGCCCAGTCCGGGGCAGCCATCTTC>3)(SEQ.ID.NO. 12) was used instead of BB28 and the PCR reaction with annealing temperature of 50°C was used for cloning. One of two clones sequenced was found to have the correct sequence.

The substitution mutation T148C was constructed using an analogous protocol but employing a different cloning strategy. The mutagenic forward oligonucleotide BB30 (5>GGGCAGATCTT CAAGCAGACCTACAGCAAGTTCGACTGCAACTCACACAAC>3)(SEQ.ID.NO. 13) was used in PCR with the non-mutagenic reverse primer BB33 (5>CGCGGTACCCGGGATCCGATTAGAAT CCACAGCT>3)(SEQ.ID.NO. 14) which anneals to the most 3' end of the GH coding sequence and spans the *Bam* HI site immediately downstream. PCR was performed as described above with the exception that the annealing temperatures used were 46, 51 and 56°C. Following PCR and gel analysis as described above the 46 and 51°C reactions were pooled for cloning. These were digested with *Bam* HI and *Bgl* II and the resulting 188 bp fragment was gel-purified and cloned into pBBT111 which had been digested with *Bam* HI and *Bgl* II, treated with calf alkaline phosphatase (Promega) according to the vendor protocols, and gel-purified. Transformants from this ligation were analyzed by digestion with *Bam* HI and *Bgl* II to identify clones in which the 188 bp *Bam* HI-*Bgl* II mutagenic PCR fragment was cloned in the proper orientation: because *Bam* HI and *Bgl* II generate compatible ends, this cloning step is not orientation specific. Five of six clones tested were shown to be correctly oriented. One of these was sequenced and was shown to contain the desired T148C mutation. The sequence of the remainder of the 188 bp *Bam* HI-*Bgl* II mutagenic PCR fragment in this clone was confirmed as correct.

The construction of the substitution mutation S144C was identical to the construction of T148C with the following exceptions. Mutagenic forward oligonucleotide BB31 (5>GGGCAGATCTTCAAGCAGACCTACTGCAAGTTCGAC>3)(SEQ.ID.NO. 15) was used instead of BB30. Two of six clones tested were shown to be correctly oriented. One of these was sequenced and was shown to contain the desired S144C mutation. The sequence of the remainder of the 188 bp *Bam* HI-*Bgl* II mutagenic PCR fragment in this clone was also confirmed as correct.

A mutation was also constructed that added a cysteine residue to the natural carboxyterminus of GH. The construction of this mutation, termed stp192C, was carried out using the procedures described above for construction of the T148C mutein but employed different oligonucleotide primers. The mutagenic reverse oligonucleotide BB32 (5'-CGCGGTACCGGATCCTTAGCAGAAGCCACAG
 5 CTGCCCTCCAC-3') (SEQ.ID.NO. 16) which inserts a TGC codon for cysteine between the codon for the carboxy terminal phe residue of GH and the TAA translational stop codon and spans the nearby *Bam* HI site was used along with BB34 which is described above. Following PCR and gel analysis as described above the 46°C reaction was used for cloning. Three of six clones tested were shown to be correctly oriented. One of these was sequenced and was shown to contain the desired
 10 stp192C mutation. The sequence of the remainder of the 188 bp *Bam* HI- *Bgl* II mutagenic PCR fragment in this clone was confirmed as correct.

The substitution mutation S100C was constructed using mutagenic reverse oligonucleotide BB25 (5'-GTCAGAGGCGCCGTACACCAGGCAGTTGGCGAAGAC-3') (SEQ.ID.NO. 17) which alters the AGC codon for serine at amino acid residue 100 to a TGC codon encoding cysteine. BB25
 15 also spans the nearby *Nar* I site. PCR reactions using BB25 and BB34 were carried out using the PCR protocol described above for the construction of the T135C mutation. Following gel analysis of the PCR products, the product of the 50°C annealing reaction was cleaned up using the QIAquick PCR Purification Kit (Qiagen), with digested with *Pst* I and *Nar* I. The resulting 178 bp fragment was gel-purified and ligated into pBBT111 which had been digested with *Pst* I and *Nar* I and gel-
 20 purified. Plasmids isolated from transformants from this ligation were screened by digestion with *Pst* I and *Nar* I and subsequently one plasmid was sequenced to confirm the presence of the S100C mutation and the absence of any other mutations in the 178 bp *Pst* I - *Nar* I segment.

The substitution mutation A98C was constructed using the procedure described above for S100C with the following differences: the mutagenic reverse oligonucleotide BB26 (5'-GTCAG
 25 AGGCGCCGTACACCAGGCTGTTGCAGAAGACACTCCT-3') (SEQ.ID.NO. 18) was used for PCR in place of BB25 and the PCR reaction performed with an annealing temperature of 45°C was used for cloning. One clone was sequenced and found to have the correct sequence.

The substitution mutation A34C was constructed as follows. The mutagenic reverse oligo BB23 (5'-GCGCTGCAGGAATGAATACTTCTGTTCCTTTGGGATATAGCATTCCTC
 30 AAATC-3') (SEQ.ID.NO. 19) was designed to change the GCC codon for alanine at amino acid residue 34 to a TGC codon encoding cysteine and to span the *Pst* I site. This oligonucleotide was used in PCR reactions along with BB11 (5'-CCCCCTCTAGACAT ATGAAGAAGAACATCGC ATTCCTGCTGGCATCTATGTTTCGTTTCTCTATCG-3') (SEQ.ID.NO. 20) which anneals to the 5' end of the coding sequence of the stII leader sequence and spans the *Nde* I site that overlaps the
 35 initiator methionine codon.

PCR reactions were performed in 50 µl in 1X PCR buffer (Promega) containing 1.5 mM MgCl₂, 200 µM concentration of each of the four nucleotides dA, dC, dG and dT, with each oligonucleotide primer present at 0.2 µM, 1 ng of pBBT111 (described above) as template and 0.8 units of *Tac* DNA Polymerase (Promega) and 0.33 units of Pfu DNA Polymerase (Stratagene).

- 5 Reactions were performed in a Robocycler Gradient 96 thermal cycler (Stratagene). The program used entailed: 96°C for 3 minutes followed by 25 cycles of [95°C for 60 seconds, 50°C or 55°C or 60°C for 75 seconds, 72°C for 60 seconds] followed by a hold at 6°C. The PCR reactions were analyzed by agarose gel electrophoresis which showed that all three different annealing temperatures gave significant product of the expected size; ~220 bp. The 50 and 55°C reactions were pooled, "cleaned up" using the QIAquick PCR Purification Kit (Qiagen) and digested with *Nde* I and *Pst* I. The
- 10 resulting 207 bp *Nde* I- *Pst* I fragment, which includes the putative A34C mutation, was gel-purified and ligated into pBBT111, which had been digested with *Nde* I and *Pst* I, treated with alkaline phosphatase, and gel-purified. Transformants from this ligation were initially screened by digestion with *Nde* I and *Pst* I and subsequently one clone was sequenced to confirm the presence of the A34C
- 15 mutation and the absence of any additional mutations that could potentially be introduced by the PCR reaction or by the synthetic oligonucleotides. The sequenced clone was found to have the correct sequence throughout the *Nde* I – *Pst* I segment.

- The substitution mutation S43C was constructed using the protocol described above for A34C with the following differences: mutagenic reverse oligonucleotide BB24 (5'>GCGCTG
- 20 CAGGAAGCAATACTTCTGTTCCTTTGG>3')(SEQ.ID.NO. 21) was used instead of BB23. One clone was sequenced and shown to contain the correct sequence.

- The substitution mutation T3C was constructed using two sequential PCR steps. The first step created the desired mutation while the second step extended the PCR product of the first reaction to encompass a useful cloning site. The mutagenic forward oligonucleotide BB78 (5'>GCAT
- 25 CTATGTTTCGTTTTCTCTATCGCTACCAACGCT TACGCATTCCCATGCATTCCCT TATCCAG>3')(SEQ.ID.NO. 22) was designed to change the ACC codon for threonine at amino acid residue 3 to a TGC codon encoding cysteine and to span and anneal to the junction of the *stII* – *hGH* fusion gene. BB78 was used in the first step PCR along with BB33 which is described above.

- The first PCR reaction was performed in 50 µl in 1X PCR buffer (Promega) containing 1.5
- 30 mM MgCl₂, 200 µM concentration of each of the four nucleotides dA, dC, dG and dT, each oligonucleotide primer at 0.2 µM, 1 ng of pBBT111 (described above) as template and 1.5 units of *Tac* DNA Polymerase (Promega) and 0.25 units of Pfu DNA Polymerase (Stratagene). Reactions were performed in a Robocycler Gradient 96 thermal cycler (Stratagene). The program used entailed: 96°C for 3 minutes followed by 25 cycles of [94°C for 60 seconds, 60°C for 75 seconds, 72°C for 60
- 35 seconds] followed by a hold at 6°C. The PCR reaction was ethanol precipitated and the ~630 bp product was gel-purified and recovered in 20 µl 10 mM Tris-HCl (pH 8.5). An aliquot of this gel-purified fragment was diluted 100-fold and 2 µl of the diluted fragment was used as template in the second PCR step. The second PCR step employed oligonucleotides BB11 and BB33 (both described above) and used the reaction conditions employed in the first step PCR reaction. The second step

PCR reaction was analyzed by agarose gel electrophoresis and the expected ~ 670 bp fragment was observed. The PCR reaction was "cleaned up" using the QIAquick PCR Purification Kit (Qiagen) and digested with *Nde* I and *Pst* I. The resulting 207 bp *Nde* I - *Pst* I fragment, which includes the putative T3C mutation, was gel-purified and ligated into pBBT111, which had been digested with *Nde* I and *Pst* I, treated with alkaline phosphatase, and gel-purified. Transformants from this ligation were initially screened by digestion with *Nde* I and *Pst* I and subsequently one clone was sequenced to confirm the presence of the T3C mutation and the absence of any additional mutations that could potentially be introduced by the PCR reaction or by the synthetic oligonucleotides. The sequenced clone was found to have the correct sequence throughout the *Nde* I - *Pst* I segment.

The substitution mutation A105C was constructed using the technique of "mutagenesis by overlap extension" as described in general by Horton, R. M. pp251-261 in *Methods in Molecular Biology*, Vol. 15: PCR Protocols: Current Methods and Applications edited by White, B. A. (1993) Humana Press, Inc., Totowa, NJ. With this technique two separate fragments are amplified from the target DNA segment as diagrammed in Figure 1. One fragment is produced with primers a and b to yield product AB. The second primer pair, c and d, are used to produce product CD. Primers b and c introduce the same sequence change into the right and left ends of products AB and CD, respectively. Products AB and CD share a segment of identical (mutated) sequence, the "overlap", which allows annealing of the top strand of AB to the bottom strand of CD, and the converse. Extension of these annealed overlaps by DNA polymerase in a subsequent PCR reaction using primers a and d with products AB and CD both added as template creates a full length mutant molecule AD.

With the exception of the use of different oligonucleotide primers, the initial PCR reactions for the A105C construction were performed identically to those described in the construction of T3C above. The primer pairs used were: (BB27 + BB33) and (BB11 + BB79). BB11 and BB33 are described above. BB27 and BB79 are complementary mutagenic oligonucleotides that change the GCC codon for alanine at amino acid residue 105 to a TGC codon encoding cysteine. The sequence of BB27 is (5>AGCCTGGTGTAC GGCTGCTCTGACAGCAACGTC>3)(SEQ.ID.NO. 23) and the sequence of BB78 is 5>GACGTTGCTG TCAGAGCAGCCGTACACCAGGCT>3 (SEQ.ID.NO. 24). The (BB27 x BB33) and (BB11 x BB79) PCR reactions were ethanol precipitated, gel-purified, and recovered in 20 µl 10 mM Tris-HCl (pH 8.5). The preparative gel showed that the predominant product from each PCR reaction was of the expected size: ~290 bp for the (BB27 x BB33) reaction and ~408 bp for the (BB11 x BB79) reaction. These two mutagenized fragments were then "spliced" together in a subsequent PCR reaction. In this reaction 1 µl of each of gel-purified fragments from the initial reactions were added as template and BB11 and BB33 were used as primers. Otherwise, the PCR reaction was performed using the same conditions employed for the initial (BB27 x BB33) and (BB11 x BB79) reactions. An aliquot of the secondary PCR reaction was analyzed by agarose gel electrophoresis and the expected ~670 bp band was observed. The bulk of the PCR reaction was then "cleaned up" using the QIAquick PCR Purification Kit (Qiagen) and digested with *Bgl* II and *Pst* I. The resulting 276 bp *Bgl* II - *Pst* I fragment, which includes the putative A105C mutation, was gel-purified and ligated into pBBT111, which had been digested with *Bgl* II and *Pst* I, and gel-purified.

Transformants from this ligation were initially screened by digestion with *Bgl* II and *Pst* I and subsequently one clone was sequenced to confirm the presence of the A105C mutation and the absence of any additional mutations that could potentially be introduced by the PCR reaction or by the synthetic oligonucleotides. The sequenced clone was found to have the correct sequence throughout the *Bgl* II – *Pst* I segment.

For expression in *E. coli* as proteins secreted to the periplasmic space, the stII-hGH genes encoding the 11 muteins were excised from the pUC19-based pBBT111 derivatives as *Nde* I – *Xba* I fragments of ~650 bp and subcloned into the pCYB1 expression vector that had been used to express rhGH. For expression experiments, these plasmids were introduced into *E. coli* W3110.

Using procedures similar to those described here and in Example 23, one can construct other cysteine muteins of GH. The cysteine muteins can be substitution mutations that substitute cysteine for a natural amino residue in the GH coding sequence, insertion mutations that insert a cysteine residue between two naturally occurring amino acids in the GH coding sequence, or addition mutations that add a cysteine residue preceding the first amino acid, F1, of the GH coding sequence or add a cysteine residue following the terminal amino acid residue, F191, of the GH coding sequence. The cysteine residues can be substituted for any amino acid, or inserted between any two amino acids, anywhere in the GH coding sequence. Preferred sites for substituting or inserting cysteine residues in GH are in the region preceding Helix A, the A-B loop, the B-C loop, the C-D loop and the region distal to Helix D. Other preferred sites are the first or last three amino acids of the A, B, C and D Helices. Preferred residues in these regions for creating cysteine substitutions are F1, P2, P5, E33, K38, E39, Q40, Q46, N47, P48, Q49, T50, S51, S55, S57, T60, Q69, N72, N99, L101, V102, Y103, G104, S106, E129, D130, G131, P133, R134, G136, Q137, K140, Q141, T142, Y143, K145, D147, N149, S150, H151, N152, D153, S184, E186, G187, S188, and G190. Cysteine residues also can be inserted immediately preceding or following these amino acids. One preferred site for adding a cysteine residue would be preceding F1, which is referred to herein as *-1C.

One also can construct GH muteins containing a free cysteine by substituting another amino acid for one of the naturally-occurring cysteine residues in GH. The naturally occurring cysteine residue that normally forms a disulfide bond with the substituted cysteine residue is now free. The non-essential cysteine residue can be replaced with any of the other 19 amino acids, but preferably with a serine or alanine residue. A free cysteine residue also can be introduced into GH by chemical modification of a naturally occurring amino acid using procedures such as those described by Sytkowski et al. (1998), incorporated herein by reference.

Using procedures similar to those described in Examples 1 – 15, one can express the proteins in prokaryotic cells such as *E. coli*, purify the proteins, PEGylate the proteins and measure their bioactivities in an *in vitro* bioassay. The GH muteins also can be expressed in eukaryotic cells such as insect or mammalian cells using procedures similar to those described in Examples 16-19, or related procedures well known to those skilled in the art. If secretion is desired, the natural GH signal sequence, or another signal sequence, can be used to secrete the protein from eukaryotic cells.

Example 5

Cystine Addition Improves Recovery of Cysteine Mutein T3C

E. coli strain W3110 containing the T3C mutation was grown overnight in 3 ml of LB containing 100 µg/ml ampicillin. The saturated overnight culture was diluted to 0.03 O.D. at A₆₀₀ in 300 ml of LB containing 100 µg/ml ampicillin and incubated at 37°C in 2 L shake flasks. When the culture O.D. reached 0.420, 1.5 ml of 100 mM IPTG was added for a final concentration of 0.5 mM to induce expression of the recombinant protein. The induced culture was incubated at 37°C overnight (~16 h).

The induced overnight culture reached an O.D. of 3.6 at A₆₀₀ and was split into 2 x 135 ml volumes and centrifuged using a Sorval RC-5 centrifuge with a GSA rotor at 8,000rpm for 10 minutes at 4°C. The supernatants were discarded and the cell pellets subjected to osmotic shock treatment as follows. The cell pellets were resuspended to approximately 49 O.D. in 10 ml of ice cold Buffer A [20% sucrose, 10 mM Tris-HCl pH 7.5] or Buffer B [20% sucrose, 10 mM Tris-HCl pH 7.5, 5.5 mM cystine (pH adjusted to 7.5-8.0)]. The pellets were resuspended by trituration and vortexing and 1 ml of 0.25 M EDTA pH 8.0 was added to give a final concentration of ~25 mM. Resuspended cells were incubated on ice for 30 minutes and centrifuged in an SS-34 rotor at 8,500 rpm for 10 minutes at 4°C. The supernatants were discarded and the pellets resuspended by trituration and vortexing in 10ml of ice cold Buffer C [5 mM Tris-HCl pH 7.5] or Buffer D [5 mM Tris-HCl pH 7.5, 5 mM cystine (pH adjusted to 7.5-8.0)] and incubated on ice for 30 minutes. The resuspended cell pellet was centrifuged in an SS-34 rotor at 8,500 rpm for 10 minutes at 4°C and the resultant supernatant (soluble periplasmic fraction) was recovered and stored at -80°C. SDS-PAGE analysis of the various supernatants revealed that by incorporating cystine into the osmotic shock procedure, the amount of monomeric hGH species recovered in the soluble periplasmic fraction can be significantly improved. In the sample treated with cystine, we observed that a majority of the hGH ran as a single band of about 22 kDa on a non-reduced SDS-PAGE gel and that band co-migrated with pituitary hGH. In the absence of cystine, a number of different molecular weight species were observed in the monomeric range. Larger molecular weight protein aggregates are visible when the gel is developed using Western blotting.

Example 6

General Method for Expression and Purification of GH Cysteine Added Variants

A standard protocol for isolation of the hGH cysteine muteins is as follows. Cultures of W3110 *E. coli* strains containing the stII-hGH mutein plasmids were grown to saturation in LB containing 100 µg/ml ampicillin at 37°C. The overnight cultures were typically diluted to 0.025-0.030 O.D. at A₆₀₀ in LB containing 100 µg/ml ampicillin and grown at 37°C in shake flasks. When culture O.D.'s reached 0.300-0.500 at A₆₀₀, IPTG was added to a final concentration of 0.5mM to induce expression. The induced cultures were incubated at 37°C overnight. Induced overnight cultures were pelleted by centrifugation in a Sorval RC-5 centrifuge at 8,000-10,000 x g for 10 minutes at 4°C and the resultant

pellets subjected to osmotic shock based on the procedures of Koshland and Botstein (1980) or Hsiung et al., (1986) depending on the size of the culture. For osmotic shock, cell pellets were resuspended at 25-50 O.D. in 20% sucrose, 10 mM Tris-HCl pH 7.5. In certain instances 5 or 25 mM EDTA pH 8.0 and/or 5 mM cystine (pH adjusted to 7.5-8.0) were included in the resuspension buffer. The resuspended pellets were incubated on ice for 15-30 minutes and centrifuged as above. The supernatants were discarded and the pellets resuspended at 25-50 O.D. at A₆₀₀ in 5 or 10 mM Tris-HCl pH 7.5. In certain instances 5 mM EDTA pH 8.0 and / or 5 mM cystine (pH adjusted to 7.5-8.0) were included in the resuspension buffer. The resuspended pellets were incubated on ice for 30 minutes and centrifuged as above. The resulting supernatant contains the soluble periplasmic components and the pellet is comprised of insoluble periplasmic, and cell associated components.

Addition of cystine to the osmotic shock buffer resulted in significantly improved recoveries and stabilization of many of the cysteine muteins. In the presence of cystine the cysteine muteins were largely monomeric, whereas in the absence of cystine, a mixture of hGH monomers, dimers and higher order aggregates were observed when the samples were analyzed by non-reducing SDS-PAGE and Western blots. Presumably the higher order aggregates are a consequence of the added cysteine residues in the proteins since they were greatly reduced or absent with wild-type rhGH. Addition of cystine to the osmotic shock buffer largely solved this problem. We believe cystine reacts with the free cysteine residue in the muteins to form a stable mixed disulfide, thus preventing disulfide shuffling and aggregation. We have also tested a second dithiol, cystamine and have seen a similar stabilizing effect on cysteine muteins of hGH. Presumably other dithiols such as oxidized glutathione would also lead to improved recoveries of proteins containing a free cysteine.

Of the 11 hGH muteins analyzed, six expressed at levels sufficient for isolation and purification. Non-reduced SDS-PAGE analysis of the osmotic shock supernatants for the A34C, S43C, A98C, S100C and S132C muteins showed little or no protein present at the correct molecular weight. Addition of cystine did not discernibly improve recovery of these proteins. The relatively low expression levels of these muteins makes it difficult to observe improved yields. Preliminary analyses of whole cell lysates indicated that these mutant proteins were expressed, but insoluble. The T3C, A105C, T135C, S144C, T148C and stp192C muteins showed moderate to good expression levels. Five of these cysteine muteins (T3C, T135C, S144C, T148C and stp192C) showed significantly improved recoveries when cystine was present in the osmotic shock buffer. A105C was not tested in the absence of cystine. General protein purification protocols are described below.

WT-rhGH was purified by ion exchange and gel filtration chromatography. The cysteine muteins were purified by an assortment of chromatographic procedures including ion exchange, hydrophobic interaction (HIC), metal chelation affinity chromatographies (IMAC), Size Exclusion Chromatography (SEC) or a combination of these techniques. Generally, the hGH mutein was captured from the fresh osmotic shock supernatant using a Q-Sepharose fast flow resin (Pharmacia) equilibrated in 20 mM Tris-HCl, pH 8.0. The column was washed with 20 mM Tris-HCl, pH 8.0 and bound proteins eluted with a linear 10 volume increasing salt gradient from 0 to 250 mM NaCl in 20 mM Tris-HCl, pH 8.0. Fractions containing the hGH muteins were identified by SDS-PAGE and

Western blotting. These fractions were pooled and stored frozen. Alternative resins can be used to capture hGH muteins from the osmotic shock mixture. These include HIC, cation ion exchange resins or affinity resins.

For hydrophobic interaction chromatography (HIC), the Q column pool was thawed to room temperature and NaCl added to a final concentration of 2 M. The pool was loaded onto the Butyl-Sepharose fast flow resin previously equilibrated in 2 M NaCl, 20 mM sodium phosphate, pH 7.5. hGH muteins were eluted from the resin using a reverse salt gradient from 2 M to 0 M NaCl in 20 mM phosphate, pH 7.5. Fractions containing the hGH muteins were identified by SDS-PAGE and Western blotting, and pooled.

In some instances, the HIC pool was subsequently loaded directly onto a nickel chelating resin (Qiagen) equilibrated in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.5. Following a wash step, muteins were recovered using a 0 – 30 mM imidazole gradient in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.5. hGH has a high affinity for nickel, presumably through the divalent metal-binding site formed by H18, H21 and E174. As a result, hGH can be obtained in highly pure form using a metal chelation column (Maisano et al., 1989). All of the muteins tested bound tightly to the nickel column and eluted at similar imidazole concentrations (around 15 mM) as wild-type rhGH.

Example 7

Purification of T3C

The soluble periplasmic fraction prepared in the presence of cystine (Example 5) was loaded onto a 1 ml HiTrap Q-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 10 mM Tris-HCl pH 8.0 and the bound proteins were eluted with a 20 column volume linear gradient of 0-250 mM NaCl. The column load and recovered fractions were analyzed by 14% SDS-PAGE. T3C eluted at a salt concentration around 170-200 mM which was significantly later than WT- hGH. Surprisingly, T3C mutein was present in a monomeric form when loaded on the column, but was recovered predominantly as a stable disulfide linked homodimer following elution from the Q column. When reduced, the T3C mutein co-migrated on a SDS-PAGE gel with wild type hGH. Dimer formation occurred during the Q column purification step. Cystine was not present in the Q column buffer or the buffers used for the other column steps. The T3C dimer remained intact throughout any further concentration or purification procedures. The pool from the Q column was adjusted to a final NaCl concentration of 0.5 M and loaded onto 1 ml Ni-agarose column (Qiagen) previously equilibrated in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.5. Following a wash step, highly purified T3C dimers were recovered using a 0 – 30 mM imidazole gradient in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.5. T3C dimers were active in the bioassay (Example 10 and Table 1). The A105C, T135C and stp192C muteins also were recovered from the Q column as disulfide-linked dimers. It appears that certain muteins are capable of forming stable disulfide-linked dimers, presumably through the added cysteine residue, once cystine is removed. We believe monomeric forms of these proteins could be stabilized by including cystine or other dithiol compounds

and/or cysteine blocking agents in all the column buffers and/or maintaining the pH of the buffers below 7 to prevent disulfide rearrangement.

Example 8

Purification of T148C

5

E. coli strain W3110 expressing the T148C mutein was grown overnight in 3 ml of LB containing 100 µg/ml ampicillin. The saturated overnight culture was diluted to 0.025 O.D. at A_{600} in 500 ml of LB containing 100 µg/ml ampicillin and divided into 2 x 250 ml volumes and incubated at 37°C in 2 L shake flasks. When the culture O.D. reached approximately 0.35, 1.25 ml of 100 mM IPTG was added to each flask for a final concentration of 0.5 mM to induce expression of the recombinant protein. The induced culture was incubated at 37°C overnight (~16 h).

The induced overnight cultures reached an O.D of approximately 3.5 at A_{600} and were centrifuged using a Sorval RC-5 centrifuge with a GSA rotor at 8,000rpm for 10 minutes at 4°C. The supernatants were discarded and the cell pellets subjected to osmotic shock treatment as follows. The cell pellets were resuspended to approximately 43 O.D. in ice cold 20% sucrose, 10 mM Tris-HCl pH 7.5, 5.0 mM EDTA pH 8.0, 5.0 mM cystine (pH adjusted to 7.5-8.0) by trituration and vortexing. Resuspended cells were incubated on ice for 15 minutes, centrifuged in Sorval centrifuge with an SS-34 rotor at 8,500 rpm for 10 minutes at 4°C. The supernatants were discarded and the pellets resuspended to approximately 43 O.D. in ice cold 1mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 5 mM cystine (pH adjusted to 7.5-8.0) by trituration and vortexing. Resuspended cells were incubated on ice for 30 minutes and centrifuged in an SS-34 rotor at 8,500 rpm for 10 minutes at 4°C and the resultant supernatant (soluble periplasmic fraction) was recovered and stored at -80°C.

A second culture of T148C was prepared in a similar manner with the following exceptions: 1) the induced culture volume was 200 ml and reached an O.D. of approximately 4.0 at A_{600} after overnight incubation at 37°C, and 2) The cell pellet resuspensions were done at approximately 30 O.D.s at A_{600} .

The soluble periplasmic fraction from the above preparations were combined and dialyzed against 1 L of 10 mM Tris-HCl pH 8.0 overnight at 4°C. The dialysis retentate was loaded onto a 5 ml HiTrap Q-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 10 mM Tris-HCl pH 8.0 and the bound proteins were eluted with a 20 column volume linear gradient of 0-250 mM NaCl. Column fractions were analyzed by 14% SDS-PAGE (Novex, San Diego, CA.). T148C mutein eluted as a sharp peak at a salt concentration around 60-80 mM. The appropriate fractions were pooled and concentrated on Centricon 10 concentrators (Amicon, Inc., Beverly, MA). The concentrated pool was further purified on a Superdex 200 HR 10/30 column (Pharmacia Biotech Inc., Uppsala, Sweden.) equilibrated in 20 mM sodium phosphate pH 7.5, 150 mM NaCl. The column fractions were again analyzed by SDS-PAGE and the T148C mutein containing fractions were pooled. We observed that the T148C remained monomeric after recovery from the Q-Sepharose column and eluted from the Superdex column at a molecular weight similar to wild type hGH. T148C prepared by Q-Sepharose and size exclusion chromatography is referred to as Lot A.

Alternatively the Q-pool can be loaded directly onto a nickel chelating resin (Qiagen) equilibrated in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.5. Following a wash step, T148C was recovered using a 0 – 30 mM imidazole gradient in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.5. T148C protein prepared by Q-Sepharose and nickel affinity chromatography is referred to as Lot B.

5 An osmotic shock T148C supernatant was prepared in the absence of cystine and purification over a Q-Sepharose column was attempted. The identical protocol was followed as described above except cystine was absent from the osmotic shock buffers. The T148C protein product eluted from the Q column over a broad range of salt concentrations, recoveries were lower and the protein preparation was substantially less pure than the cystine treated T148C sample.

10

Example 9

Purification of S144C

E. coli strain W3110 containing the S144C mutation was grown overnight in 3 ml of LB containing 100 µg/ml ampicillin. The saturated overnight culture was diluted to 0.03 O.D.s at A₆₀₀ in 250 ml of LB containing 100 µg/ml ampicillin and incubated at 37°C in a 2 L shake flask. When the culture O.D. reached approximately 0.3, 1.25 ml of 100 mM IPTG was added for a final concentration of 0.5 mM to induce expression of the recombinant protein. The induced culture was incubated at 37°C overnight (~16 h).

20 The induced overnight culture reached an O.D of approximately 3.3 at A₆₀₀ and was centrifuged using a Sorval RC-5 centrifuge with a GSA rotor at 8,000rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet subjected to osmotic shock treatment as follows. The cell pellet was resuspended to approximately 33 O.D. in ice cold 20% sucrose, 10 mM Tris-HCl pH 7.5, 5.0 mM cystine (pH adjusted to 7.5-8.0) by trituration and vortexing and 0.25 M EDTA pH 8.0 was added to a final concentration of 25 mM. Resuspended cells were incubated on ice for 30 minutes, centrifuged in the Sorval RC-5 centrifuge with an SS-34 rotor at 8,500 rpm for 10 minutes at 4°C. The supernatants were discarded and the pellets resuspended to approximately 33 O.D.s in ice cold 5 mM Tris-HCl pH 7.5, 5 mM cystine (pH adjusted to 7.5-8.0) by trituration and vortexing. Resuspended cells were incubated on ice for 30 minutes and centrifuged in an SS-34 rotor at 8,500 rpm for 10 minutes at 4°C and the resultant supernatant (soluble periplasmic fraction) was recovered and stored at -80°C.

30 The soluble periplasmic fraction from the above preparation was loaded onto a 5 ml HiTrap Q-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 10 mM Tris-HCl pH 8.0 and the bound proteins were eluted with a 20 column volume linear gradient of 0-250 mM NaCl. Column fractions were analyzed by 14% SDS-PAGE (Novex, San Diego, CA.) and S144C mutein-containing fractions, 125-150 mM NaCl, were pooled and frozen. The S144C mutein was monomeric.

Example 10**Bioactivities of hGH Cysteine Muteins**

The biological activities of the purified T3C, S144C and T148C cysteine muteins were measured using the cell proliferation assay described in Example 1. Protein concentrations were determined using a Bradford assay. All three muteins were biologically active. All of the muteins reached the same level of maximum stimulation as pituitary hGH, within the error of the assay. The mean EC₅₀s for the T3C, S144C and T148C muteins were similar to that of pituitary hGH and rhGH (Table 1). Two independent preparations of the T148C mutein and one preparation each of the S144C and T3C muteins (all prepared in the presence of cystine) have been assayed multiple times (Table 1). The partially purified A105C, T135C and stp192C muteins also have been assayed and are biologically active.

Example 11**General Methods for Pegylation and Purification of Cysteine Muteins**

GH muteins can be PEGylated using a variety of cysteine-reactive PEG-maleimide (or PEG-vinylsulfone) reagents that are commercially available. Generally, methods for PEGylating the proteins with these reagents will be similar to those described in WO 9412219 (Cox and McDermott) and WO 9422466 (Cox and Russell), both incorporated herein by reference, with minor modifications. The recombinant proteins are generally partially reduced with dithiothreitol (DTT), Tris (2-carboxyethyl) phosphine-HCl (TCEP) or some other reducing agent in order to achieve optimal PEGylation of the free cysteine. The free cysteine is relatively unreactive to cysteine-reactive PEGs unless this partial reduction step is performed. The amount of reducing agent required to partially reduce each mutein can be determined empirically, using a range of reducing agent concentrations at different pHs and temperatures. Reducing agent concentrations typically vary from 0.5 equal molar to 10-fold excess. Preferred temperatures are 4°C to 37°C. The pH can range from 6.5 to 9.0 but is preferably 7.5 to 8.5. The optimum conditions will also vary depending on the reductant and time of exposure. Under the proper conditions, the least stable disulfides (typically intermolecular disulfides and mixed disulfides) are disrupted first rather than the more thermodynamically stable native disulfides. Typically, a 5-10 fold molar excess of DTT for 30 minutes at room temperature is effective. Partial reduction can be detected by a slight shift in the elution profile of the protein from a reversed-phase column. In the case of a dimeric hGH, a shift in molecular weight is visible by SDS-PAGE analysis run under non-reducing condition. Care must be taken not to over-reduce the protein and expose additional cysteine residues. Over-reduction can be detected by reversed phase-HPLC (the over-reduced protein will have a retention time similar to the fully reduced and denatured protein) and by the appearance of GH molecules containing two PEGs following the PEGylation reaction (detectable by a molecular weight change on SDS-PAGE). Wild type GH can serve as a control since it should not PEGylate under conditions that do not reduce the native intramolecular disulfides. Excess reducing agent can be removed prior to PEGylation by size exclusion chromatography or by

dialysis. TCEP need not be removed before addition of the PEGylation reagent as it does not contain a free thiol group. The partially reduced protein can be reacted with various concentrations of PEG-maleimide or PEG-vinylsulfone (typically PEG: protein molar ratios of 1:1, 5:1, 10:1 and 50:1) to determine the optimum ratio of the two reagents. PEGylation of the protein can be monitored by a molecular weight shift for example, using SDS-PAGE. The lowest amount of PEG that gives significant quantities of mono-pegylated product without giving di-pegylated product is typically considered optimum (80% conversion to mono-pegylated product is generally considered good). Generally, mono-PEGylated protein can be purified from non-PEGylated protein and unreacted PEG by size-exclusion, ion exchange, affinity, reversed phase, or hydrophobic interaction chromatography. Other purification protocols such as 2-phase organic extraction or salt precipitation can be used. The purified PEGylated protein can be tested in the cell proliferation assay described in Example 1 to determine its specific activity.

Experiments can be performed to confirm that the PEG molecule is attached to the protein at the proper site. This can be accomplished by chemical or proteolytic digestion of the protein, purification of the PEGylated peptide (which will have a large molecular weight) by size exclusion, ion exchange or reversed phase chromatography, followed by amino acid sequencing. The PEG-coupled amino acid will appear as a blank in the amino acid sequencing run.

Example 12

Preparation and Purification of PEG-T3C

A preliminary titration study was performed to determine appropriate reducing agent and PEG reagent concentrations and to avoid over-reduction of the protein. We monitored partial reduction of the protein by conversion of dimer to non-reduced monomer species on non-reduced SDS-PAGE. One μ g aliquots of purified T3C dimer were incubated with increasing concentrations of TCEP for 60 minutes at room temperature. The reactions were immediately analyzed by non-reducing SDS-PAGE. The amount of TCEP that yielded significant amounts of monomer T3C without overreducing and denaturing the protein was used for subsequent experiments. TCEP is a convenient reducing agent for small scale experiments because it does not interfere with the PEGylation reaction; thus the protein:TCEP mixture did not have to be dialyzed prior to PEG addition. At a larger scale inexpensive reducing agents such as DTT are preferred for reducing proteins. Generally, the protein is treated with a reducing agent for an optimal amount of time. The pH of the reaction is then adjusted to 6.5 or below to limit disulfide rearrangements. The reducing agent is removed by dialysis or liquid chromatography. The pH is then readjusted to greater than 6.5 or preferably 7.5 to 8.5 and the PEG reagent is added.

The titration experiments at pH 7.5 indicated that a five-fold molar excess of TCEP for 60 minutes at room temperature converted the majority of the T3C dimer species into properly disulfide-bonded monomer without over reducing the protein. Control experiments indicated that, as expected, the T3C dimer needed to be reduced with TCEP to be PEGylated. These reaction conditions were then scaled to 100 μ g reaction scale. A 10-fold molar excess of 5 kDa maleimide-PEG (Fluka) was

added to the T3C:TCEP mixture after 10 minutes and the PEGylation reaction was allowed to proceed for 60 minutes at room temperature. The sample was loaded quickly onto a 1 ml Q-Sepharose column equilibrated in 20 mM Tris-HCl, pH 8.0. The column was washed with 20 mM Tris-HCl, pH 8.0 and bound proteins eluted with a linear 10 volume increasing salt gradient from 0 to 250 mM NaCl in 20 mM Tris-HCl, pH 8.0. Fractions containing mono-PEGylated T3C (a single PEG molecule attached to the T3C monomer) were identified by SDS-PAGE and Western blotting. These fractions were pooled and stored frozen. The presence of the PEG moiety decreases the protein's affinity for the resin, allowing the PEGylated protein to be separated from the non-PEGylated protein.

10

Example 13

PEGylation of S144C and other Cysteine Muteins

A preliminary titration study was also performed for S144C to determine appropriate reducing agent, PEG reagent concentrations and to avoid over-reduction of the protein as described in Example 12 for T3C. The larger scale PEGylation was carried out at pH 7.5 at room temperature for 2 h using a 2-fold molar excess of TCEP and a 10-fold molar excess of 5 kDa maleimide-PEG. SDS-PAGE analysis of the reaction mixture showed some species with two or more PEGs present. These were separated from the mono-PEGylated S144C using a Q-Sepharose column as described in Example 12. Separately we performed a PEGylation reaction using 5 kDa vinylsulfone-PEG (Fluka) which resulted in monopegylated S144C under identical reducing conditions.

15

We have also performed small scale PEGylation reactions on T148C, stp192C and T135C, all of which yielded mono-PEGylated protein with 5 kDa maleimide-PEG and/or 5 kDa vinylsulfone-PEG.

20

Example 14

Bioactivity of the PEG-T3C and PEG-S144C hGH Proteins

The biological activity of the PEG-T3C and PEG-S144C proteins were measured in the cell proliferation assay (Example 1). The PEG-T3C protein showed a similar dose-response curve as pituitary hGH and non-PEGylated T3C protein and reached the same level of maximal stimulation. The mean EC₅₀ value for PEG-T3C was 1.6 ng/ml (0.07 nM) (values of 1.3, 1.5, 1.7, 1.8 ng/ml in four experiments). Bioactivity of the PEG-T3C protein is at least 100-fold greater than that of rhGH that has been PEGylated using non-specific NHS-PEG reagents (EC₅₀ of 440 ng/ml (20 nM) as described in Clark et al., (1996).

25

The mean EC₅₀ value for PEG-S144C was 43 ng/ml (2 nM) (40 and 45 ng/ml in two experiments). Bioactivity of the PEG-S144C protein is approximately 10-fold greater than that of rhGH that has been PEGylated using non-specific NHS-PEG reagents (EC₅₀ of 440 ng/ml (20 nM); Clark et al., 1996).

30

Example 15**Construction of Disulfide-linked Trimers and
Disulfide-linked Higher Order Multimers of hGH**

5 Additional hGH variants having more than one "free" cysteine could be constructed and used to create higher order disulfide-linked multimers of hGH. These multimers could include trimers, tetramers, pentamers, hexamers, septamers, octamers, and any higher order multimers. For example, an hGH variant having two "free" cysteine residues could be constructed by using recombinant DNA technology to recombine *in vitro* DNA plasmid vectors carrying individual "free" cysteine mutations. 10 Alternatively, mutagenesis of an hGH cysteine variant could be employed to add an additional "free" cysteine mutation. Further iterations of either of these two procedures could be used to construct hGH variants having three or more "free" cysteines.

An hGH variant having two free cysteine residues could be used to generate hGH trimers and higher order multimers as follows. Such a variant would be expressed in *E. coli* and recovered as a 15 monomer in the supernatant of an osmotic shock lysate as disclosed in Examples 5 – 9 herein. Subsequent processing steps could then be employed to induce di-sulfide bond formation, e.g. Q Sepharose chromatography as described in Examples 6 – 9 herein. Under such conditions some hGH variants having one free cysteine, such as T3C and stp192C, are converted virtually quantitatively to disulfide-linked dimers. Under the same or similar conditions intermolecular disulfide formation by 20 an hGH variant having two free cysteines, e. g. a double mutant that combined T3C and stp192C, would result in a polymerization of hGH molecules and the chain length of such polymers would in principle be unlimited. The chain length could be limited and to some extent controlled by addition to the polymerization reaction of hGH molecules having only one free cysteine such as the T3C variant and / or the stp192C variant. Disulfide bond formation between the growing polymer and a molecule 25 having only one free cysteine will "cap" or prevent further extension of one of the two polymerization sites in the nascent polymer. A subsequent reaction of a second hGH molecule that has only one free cysteine with the other polymerization site of that nascent polymer terminates polymerization and fixes the length of that polymeric molecule. The average polymer length could be controlled by the stoichiometry of the reactants, i.e. the ratio of hGH molecules with two free cysteines to hGH 30 molecules with one free cysteine. Average shorter polymers would be favored by lower ratios and average longer polymers would be favored by higher ratios. More complex "branched" polymers could be constructed from reactions involving hGH variants with 3 or more free cysteines with hGH variants having only one free cysteine.

Discrete size classes of certain polymers could subsequently be purified by 35 chromatographic methods such as size exclusion chromatography, ion exchange chromatography, hydrophobic interaction chromatography, and the like. Similar procedures to those described for GH could be used to create disulfide-linked dimers and higher order multimers of EPO and alpha interferon.

EXAMPLE 16

Cloning, Expression and Purification of Baculovirus (BV)-Expressed Recombinant Human Erythropoietin (rEPO)

A. Cloning a cDNA encoding EPO. A cDNA encoding human EPO was cloned by PCR using
5 forward primer BB45 (5' > CCCGGAT CCATGGGGGTGCACGAATGTCCTG >3)(SEQ.ID.NO.
25) and reverse primer BB47 (5' > CCCGA ATTCTATGCCAGGTGGACACACCTG
>3)(SEQ.ID.NO. 26). BB45 anneals to the DNA sequence encoding the initiator methionine and
amino terminal portion of the EPO signal sequence and contains a *Bam* HI site for cloning purposes
(Jacobs et al., 1985; Lin et al., 1985). BB47 anneals to the 3' untranslated region of the EPO mRNA
10 immediately downstream of the translational stop signal and contains an *Eco* RI restriction site for
cloning purposes. Total RNA isolated from the human liver cell line Hep3B was used in first strand
synthesis of single-stranded cDNA for PCR.

For preparation of total cellular RNA, Hep3B cells (American Type Culture Collection) were
grown in Delbecco's Modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum
15 (FBS). EPO expression was induced by treating the cells for 18 h with 130 μ M Deferoxamine or 100
 μ M cobalt chloride. Both compounds have been shown to induce EPO mRNA and protein expression
in Hep 3B cells (Wang and Semenza, 1993). RNA was isolated from the cells using an RNeasy Mini
kit (Qiagen, Inc.), following the manufacturer's directions. Approximately 320 μ g of total RNA was
isolated from 1.4×10^7 cells treated with cobalt chloride and 270 μ g of total RNA isolated from 1.4
20 $\times 10^7$ cells treated with Deferoxamine. First strand synthesis of single-stranded cDNA was
accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer
Mannheim Corp and random hexamers were used as the primer. Subsequent PCR reactions using the
products of the first strand syntheses as templates were carried out with primers BB45 and BB47. The
expected ~ 600 bp PCR product was observed when reaction products were run out on an agarose gel.
25 Both RNA preparations yielded an EPO PCR product. The PCR product was digested with *Bam* HI
and *Eco* RI and cloned into vector pUC19 that had been cut with *Bam* HI and *Eco* RI and treated with
alkaline phosphatase. DNA sequencing identified a clone containing the correct coding sequence for
the EPO gene. This plasmid was designated pBBT131 and used in the construction of EPO variants
by site directed mutagenesis as described in Example 17.

30

B. Expression of BV rEPO in Insect Cells

For expression in insect cells the EPO cDNA in pBBT131 was modified at both the 5' and 3'
ends. At the 5' end, the sequence CAAA was added immediately upstream of the initiator ATG to
enhance translation. This sequence comprises a proposed consensus translational initiation sequence
35 for baculovirus (Ayres et al., 1994; Ranjan and Hasnain, 1995). At the 3' end, DNA encoding the 8
amino acid FLAG epitope sequence (asp-tyr-lys-asp-asp-asp-lys)(SEQ.ID.NO. 27) was added to
provide a purification system. The FLAG epitope was fused to the EPO gene via a flexible linker: ser-
gly-gly-ser-gly-gly-ser (SEQ.ID.NO. 28). These modifications were made via PCR using
oligonucleotide primers that incorporated the desired additions to the EPO sequence. Oligonucleotide

primer BB63 (5' > CGCGGATCCAAAATGGGGGTGCAC GAATGTCCT>3')(SEQ.ID.NO. 29) was used to modify the 5' end of the gene. BB63 adds the CAAA sequence upstream of the ATG, anneals to the DNA sequence encoding the initiator methionine and amino terminal portion of the EPO signal sequence, and contains a *Bam* HI site for cloning purposes. The linker and FLAG sequences were added in two sequential PCR reactions using reverse primers BB60 (5' > GTCTTTGTAGTCCGAGCCTCCGCTTCCGCCGATCT GTCC CCTGTCCTGCA >3')(SEQ.ID.NO. 30) and BB61 (5' > CGCGAATTCTTATTTATCGTCATCGTCTTTGTAGT CCGAGCCTCC >3')(SEQ.ID.NO. 31). BB60 anneals to 3' of the EPO coding sequence and contains the fused peptide linker sequence and a portion of the FLAG sequence. BB61 overlaps a segment of the BB60 sequence annealing to the junction of the linker-FLAG sequence and adds the remainder of the FLAG sequence followed by a translational stop codon (TAA) and an *Eco* RI site for cloning purposes. The modified EPO cDNA was cloned as a *Bam* HI – *Eco* RI fragment into pUC19 and the DNA sequence of this construct was confirmed. The resulting plasmid was designated pBBT132 and used in the construction of EPO variants as described in Example 17. For expression in baculovirus, the "FLAG-tagged" EPO cDNA was excised from pBBT132 as ~ 630 bp *Bam* HI – *Eco* RI fragment, gel purified, and cloned into the baculovirus transfer vector pBlueBac4.5 (Invitrogen) which had been cut with *Bam* HI and *Eco* RI and treated with alkaline phosphatase. One clone was picked for further use and designated pBBT138.

pBBT138 DNA was used to cotransfect *Spodoptera frugiperda* derived cell line Sf 9 along with linearized (*Bsu*36 I digested) Bac-N-Blue™ (Invitrogen Corporation) baculovirus DNA. The Bac-N-Blue™ genome is engineered so that formation of plaque-forming viral particles requires recombination between the linear Bac-N-Blue™ DNA and the pBlueBac4.5 vector, resulting in the incorporation of the cloned EPO gene into the baculovirus genome. This obligate recombination also results in incorporation of a functional β -galactosidase gene into the baculovirus genome. The cotransfection was performed according to the Invitrogen "Bac-N-Blue™ Transfection Kit" protocols using 2×10^6 Sf 9 cells to generate a ~ 1 ml supernatant. Dilutions of this supernatant were assayed on Sf 9 cells at 27° C for blue-plaque formation. Ten blue plaques were picked and subcultured. Each plaque was used to inoculate 2.5×10^6 Sf 9 cells in a T25 flask containing 5 ml of Grace's Insect Media supplemented with 10% FBS. After 5 days the supernatants from these infected cells (the "P1" stocks) were collected and assayed by Western Blot for EPO expression. The ten resultant supernatants were prepared in SDS sample buffer with the addition of 1% β mercaptoethanol (BME) and electrophoresed on precast 14% Tris-glycine polyacrylamide gels (Novex). Uninfected SF9 cell supernatant was included as a negative control. Following electrophoresis, the proteins were transferred onto 0.45 μ m nitrocellulose (Novex). The nitrocellulose membrane was blocked in Tris Buffered Saline (TBS) with 0.05% Tween 20 and 4% powdered milk (blocking buffer). Anti-FLAG M2 mouse IgG₁ monoclonal antibody (Eastman KODAK) was used at 1:1500 or 1:2500 dilution in blocking buffer and the blot routinely incubated overnight at 4°C. Alkaline phosphatase conjugated Sheep Anti-Mouse IgG₁ (The Binding Site Limited) was diluted 1:1000 in blocking buffer and the blot incubated for 1 hour at room temperature. The Western blot was developed using NBT/BCIP

color development substrate (Promega). Nine of the ten isolates were positive for EPO expression. The molecular weight of the BV rEPO protein was approximately 30 kDa under reducing conditions and consisted on one major and one to two minor bands in this molecular weight range, which is consistent with a variably glycosylated protein.

5 Two of the positive supernatants were tested in the *in vitro* EPO bioassay described in Example 16.D below. Both supernatants stimulated proliferation of the EPO-dependent cell line in a dose-dependent manner, indicating that they contained active EPO. Control supernatants of mock infected Sf 9 cells and the one baculovirus supernatant that was negative for EPO expression by Western blot showed no detectable activity in the EPO bioassay.

10 In order to produce and purify larger amounts of wild type BV rEPO, one positive recombinant baculovirus, termed bvBBT138A, was chosen for further amplification. A 500 ml high titer viral stock was prepared by subculturing the P1 stock of isolate 138A at 27° C in a 500 ml spinner flask culture of Sf 9 cells in Grace's Insect Media supplemented with 10% FBS. Grace's Insect Media contains approximately 100 µM L-cystine. The supernatant from this culture was
15 harvested after 7 days and found to have a titer of ~ 10⁸ plaque-forming-units / ml. An aliquot of this lysate, termed the "P2" stock, was subsequently used to infect a 500 ml culture for larger scale production of wild type BV rEPO. A 500 ml culture of Sf 9 cells in Grace's Insect Media supplemented with 10% FBS was grown in a spinner flask to a titer of 1.0 x 10⁶ / ml and then infected with bvBBT138A at a multiplicity of infection of 1. After 3 days the supernatant from this
20 culture was harvested and wild type BV rEPO-138A protein purified as described in Example 16.C.

C. Affinity Purification of Wild Type Baculovirus-Produced rEPO

The cell supernatant was clarified by centrifugation and 0.2µM filtration. Expression of wild type BV rEPO was confirmed by Western blot analysis. Wild type BV rEPO was purified in a single
25 step procedure using Anti-FLAG M2 Affinity Gel (Eastman KODAK). Briefly, 5ml of the M2 affinity gel was washed with 5 column volumes of 50mM Tris pH 7.4, 150mM NaCl (TBS), 5 column volumes of 0.1M glycine pH 3.5, then equilibrated in TBS. The clarified baculoviral cell supernatant was adjusted to 150mM NaCl and the equilibrated resin was added. Batch loading was allowed to continue at 4°C overnight on a roller bottle apparatus. After overnight incubation, the resin was
30 recovered using a Pharmacia XK 16/20 FPLC column and washed with TBS until the A280 reached baseline. The bound protein was eluted with 0.1M glycine pH 3.5 and fractions were collected and neutralized with 1.0M Tris pH 9.0. Column fractions were prepared in SDS-PAGE sample buffer with the addition of 1% BME when desirable and electrophoresed on precast 14% Tris-glycine polyacrylamide gels. Fractions from the M2 affinity column that contained most of the BV rEPO were
35 pooled and concentrated on a Centricon 10 spin concentrator (Amicon). The final yield of wild type BV rEPO, as determined using a Bradford protein assay kit (BIO-RAD Laboratories, Inc.) and bovine serum albumin (BSA) as the standard, was approximately 360µg. The protein was estimated to be greater than 90% pure by Coomassie Blue staining of SDS gels.

D. *In Vitro* Bioactivity of Wild Type Baculovirus rEPO

A cell proliferation assay using the human UT7/epo cell line (Komatsu et al., 1991) was developed to measure bioactivity of wild type BV rEPO. The human UT7/epo cell line was obtained from Dr. F. Bunn of Harvard Medical School. This cell line is dependent upon EPO for cell proliferation and survival (Boissel et al., 1993). The cells were maintained in Iscove's Modified Delbecco's Media (IMDM) supplemented with 10% FBS, 50 units/ml penicillin, 50 µg/ml streptomycin and 1 unit/ml rEPO (CHO (Chinese Hamster Ovary) cell-expressed; purchased from R&D Systems, Inc.). For bioassays, the cells were washed three times with IMDM media and resuspended at a concentration of 1×10^5 cells/ml in IMDM media containing 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. Fifty µl (5×10^3 cells) of the cell suspension were aliquotted per test well of a flat bottom 96 well tissue culture plate. Serial 3-fold dilutions of the protein samples to be tested were prepared in phenol red-free IMDM media containing 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. Fifty µl of the diluted protein samples were added to the test wells and the plates incubated at 37°C in a humidified 5% CO₂ tissue culture incubator. Protein samples were assayed in triplicate wells. After 60-72 h, 20 µl of CellTiter 96 Aqueous One Solution Reagent (Promega Corporation) was added to each well and the plates incubated at 37°C in the tissue culture incubator for 1-4 h. Absorbance of the wells was read at 490 nm using a microplate reader. Control wells contained media but no cells. Mean absorbance values for the triplicate control wells were subtracted from mean values obtained for each set of triplicate test wells. Serial dilutions of CHO cell-expressed rEPO or wild type BV rEPO were analyzed in parallel.

The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-dependent increase in cell number and absorbance values. Absorbance is proportional to cell number up to values of 2.0 (Promega Corporation product specifications). In the absence of rEPO, the majority of UT7/epo cells die, giving absorbance values less than 0.1. Commercial CHO cell-expressed rEPO and wild type BV rEPO prepared by us reached the same maximal level of stimulation, within the error of the assay, and had similar mean EC₅₀s in the bioassay of approximately 0.4-0.5 ng/ml (Table 2). EC₅₀ values for these proteins ranged from 0.21 to 0.65 ng/ml in assays performed on different days (Table 2); therefore comparisons between protein samples were made on samples analyzed on the same day. These EC₅₀ values agree with values reported in the R&D Systems specifications for CHO rEPO (0.05 – 0.1 unit/ml or approximately 0.4-0.8 ng/ml).

EXAMPLE 17**Construction, Expression, Purification and Bioactivity of EPO Cysteine Muteins****A. Construction of EPO Cysteine Muteins.**

Eight mutant EPO genes were constructed using site-directed PCR-based mutagenesis procedures similar to those used to construct the Growth Hormone muteins described in Example 4 (Innis et al., 1990; Horton et al., 1993). We constructed four muteins at or near the two N-linked glycosylation sites in the A-B loop (N24C, T26C, N38C and T40C), two muteins at or near the N-linked glycosylation site in the B-C loop (N83C and S85C), one mutein at the O-linked glycosylation site in

the C-D loop (S126C) and one mutein (*167C), that adds a cysteine between the natural carboxyterminal R166 residue and the 15 amino acid carboxyterminal extension consisting of the peptide linker and FLAG sequences. The template for the mutagenic PCR reactions was plasmid pBBT131, in which the unmodified EPO gene is cloned as an *Bam* HI – *Eco* RI fragment into pUC19.

5 PCR products were digested with appropriate restriction endonucleases, gel-purified and ligated with pBBT132 vector DNA that had been cut with appropriate restriction enzymes, alkaline phosphatase treated, and gel-purified. As detailed above, pBBT132 is a pUC19 derivative carrying the cloned modified (FLAG tagged) EPO gene. Transformants from these ligations were grown up and plasmid DNAs isolated and sequenced. The sequence of the entire cloned mutagenized PCR fragment was

10 determined to verify the presence of the mutation of interest, and the absence of any additional mutations that potentially could be introduced by the PCR reaction or by the synthetic oligonucleotide primers.

The cysteine substitution mutation N24C was constructed as follows. The mutagenic forward oligonucleotide BB64 (5' > GAGGCCAAGGAGGCCGAGTGTATCACGACGGGCTGTGCT

15 >3)(SEQ.ID.NO. 32) was designed to change the codon AAT for asparagine at position 24 to a TGT encoding cysteine and to span the nearby *Sty* I site. This oligo was used in PCR with the reverse, non-mutagenic, primer BB47 (5' > CCCGAATTCTGGTGGATATGCCCAGGTGGAC >3)(SEQ.ID.NO. 33) which anneals to DNA sequences 3' to the coding sequence of EPO. A 50 µl PCR reaction was performed in 1X Promega PCR buffer containing 1.5 mM MgCl₂, each primer at 0.2µM, each of

20 dATP, dGTP, dTTP and dCTP at 200 µM, 1 ng of template plasmid pBBT131 (described in Example 16), 1.5 units of Taq Polymerase (Promega), and 0.25 units of Pfu Polymerase (Stratagene). Reactions were performed in a Robocycler Gradient 96 thermal cycler (Stratagene). The reaction program entailed: 96°C for 3 minutes, 25 cycles of [95°C for 1 minute, 60°C for 1.25 minutes, 72°C for 1 minute] followed by a hold at 6°C. A 5 µl aliquot of the PCR reaction was analyzed by agarose

25 gel electrophoresis and found to produce a single fragment of the expected size ~ 470 bp. The remainder of the reaction was "cleaned up" using the QIAquick PCR Purification (Qiagen) according to the vendor protocol, digested with *Sty* I and *Bsr* GI (New England BioLabs) according to the vendor protocols, ethanol-precipitated, resuspended in 20 µl of 10 mM Tris-HCl pH 8.5 and run out on a 2% agarose gel. The ~400 bp *Sty* I - *Bsr* GI fragment of interest was gel purified using a

30 QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol. This fragment was ligated with pBBT132 (described in Example 16) that had been cut with *Sty* I and *Bsr* GI, treated with calf intestinal alkaline phosphatase (New England BioLabs) and gel purified. The ligation reaction was used to transform *E. coli* and plasmids from resulting transformants were sequenced to identify a clone containing the N24C mutation and having the correct sequence throughout the remainder of

35 the ~400 bp *Sty* I - *Bsr* GI segment.

The substitution mutation T26C was constructed and its sequence verified using the protocol described above for N24C except that mutagenic forward oligo BB65 (5' > GAGGCCAAGGA GGCCGAGAAATCTGTACGGGCTGTGCT >3)(SEQ.ID.NO.34) was used instead of BB64.

The substitution mutation N38C was constructed using the technique of "mutagenesis by overlap extension" as described in Example 4. With the exception of the use of different oligonucleotide primers, the initial, or "primary" PCR reactions for the N38C construction were performed identically to those described in the construction of N24C above. The primer pairs used were (BB66 + BB47) and (BB67 + BB45). BB47 is described above. The forward, non-mutagenic, primer BB45 (5' > CCCGGATCCATGGGGGTGCACGAATGTCCTG >3)(SEQ.ID.NO. 35) anneals to the EPO sequence encoding the first seven amino acids of EPO. BB66 and BB67 are complementary mutagenic oligonucleotides that change the AAT codon for N38 to a TGT codon for cysteine. The sequence of BB66 is (5' > AGCTTGAATGAGTGTATCACTGTCCCAGACACC >3)(SEQ.ID.NO. 36) and the sequence of BB67 is (5' > GGTGTCTGGGACAGTGATACACTCATT CAAGCT >3)(SEQ.ID.NO. 37). The (BB66 x BB47) and (BB67 x BB45) PCR reactions gave products of the expected sizes: ~420 bp for (BB66 x BB47) and ~220 bp for (BB67 x BB45). The PCR products were ethanol - precipitated, gel-purified and recovered in 20 µl 10 mM Tris-HCl as detailed above. These two mutagenized fragments were then "spliced" together in the subsequent, or "secondary" PCR reaction. In this reaction 1 µl of each of the gel-purified PCR products of the primary reactions were used as template and BB45 and BB47 were used as primers. Otherwise, the reaction conditions identical to those used in the primary reactions. An aliquot of the secondary PCR was analyzed by agarose gel electrophoresis and the expected band of ~630 bp was observed. The bulk of the secondary PCR reaction was "cleaned up" using the QIAquick PCR Purification (Qiagen) according to the vendor protocol, digested with *Sly* I and *Bsr* GI (New England BioLabs) according to the vendor protocols, ethanol-precipitated, resuspended in 20 µl of 10 mM Tris-HCl pH 8.5 and run out on a 2% agarose gel. The ~400 bp *Sly* I - *Bsr* GI fragment of interest was gel purified using a QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol. This fragment was ligated with pBBT132 (described in Example 16) that had been cut with *Sly* I and *Bsr* GI, treated with calf intestinal alkaline phosphatase (New England BioLabs) and gel purified. The ligation reaction was used to transform *E. coli* and plasmids from resulting transformants were sequenced to identify a clone containing the N38C mutation and having the correct sequence throughout the ~400 bp *Sly* I - *Bsr* GI segment.

The substitution mutation T40C was constructed and sequence verified using the procedures described above for N38C except that complementary mutagenic primers BB68 (5' > AGCTTG AATGAGAATATCTG TGTCCCAGACACC >3)(SEQ.ID.NO. 38) and BB69 (5' > GGTGTCTGGGACACAGATATTCTC ATTCAAGCT >3)(SEQ.ID.NO. 39), which change the ACT codon for T40 to a TGT codon for cysteine, replaced BB66 and BB67 respectively in the primary PCR reactions.

The substitution mutation N83C was constructed and sequence verified using the procedures described above for N38C except that complementary mutagenic primers BB70 (5' > GCCCTGT TGGTCTGCTCTTCCCAGCCGTGGGAGCCCCTG >3)(SEQ.ID.NO. 40) and BB71 (5' > CAGGGGCTCCACGGCTGG GAAGAGCAGACCA ACAGGGC >3)(SEQ.ID.NO. 41), which change the AAC codon for N83 to a TGC codon for cysteine, replaced BB66 and BB67, respectively,

in the primary PCR reactions. The sizes of the products of the primary PCR reactions were also different. The (BB70 x BB47) reaction gave, as predicted, a product of ~300 bp and the (BB71 x BB45) reaction gave, as predicted, a product of ~360 bp.

The substitution mutation S85C was constructed and sequence verified using the procedures described above for N38C except that complementary mutagenic primers BB72 (5' GCC CTGTTGGTCAAC TCTTGCCAGCCGTGGGAGCCCCCTG >3)(SEQ.ID.NO. 42) and BB73 (5' CAGGGGCTCCACG GCTGGCAAGAGTTGACCAACAGGGC >3)(SEQ.ID.NO. 43), which change the TCC codon for S85 to a TGC codon for cysteine, replaced BB66 and BB67 respectively in the primary PCR reactions. The sizes of the products of the primary PCR reactions were also different. The (BB72 x BB47) reaction gave, as predicted, a product of ~300 bp and the (BB73 x BB45) reaction gave, as predicted, a product of ~360 bp.

The substitution mutation S126C was constructed using the procedures described above for N38C except that complementary mutagenic primers BB74 (5' CCAGATGCGGCCTGTGCTGCTGCCACTC >3)(SEQ.ID.NO. 44) and BB75 (5' GAGTGGAGCAGCACAGGCCGCATCTGG >3)(SEQ.ID.NO. 45), which change the TCA codon for S126 to a TGT codon for cysteine, replaced BB66 and BB67 respectively in the primary PCR reactions. The sizes of the products of the primary PCR reactions were also different. The (BB74 x BB47) reaction gave, as predicted, a product of ~175 bp and the (BB75 x BB45) reaction gave, as predicted, a product of ~480 bp.

A mutation was also constructed that added a cysteine following the carboxyterminal amino acid of the EPO coding sequence. This mutant, termed *167C was constructed as follows. A PCR reaction was carried out under the conditions described above for the construction of the N24C mutant, but employing oligonucleotides BB45 (see above) and reverse mutagenic oligonucleotide BB77 (5' TTTGTAGTCCGAG CCTCCGCTTCCGCCCCGAACA TCTGTCCCCTGTCCTGCA>3)(SEQ.ID.NO. 46) and using 2.5 units of Taq Polymerase and 0.5 units of Pfu Polymerase. BB77 anneals to the terminal 21 residues of EPO coding sequence and adds a TGT codon for cysteine following the AGA codon for R166, which is the terminal amino acid in the EPO coding sequence. BB77 also adds sequences encoding the seven amino acid linker -ser-gly-gly-ser-gly-gly-ser- (SEQ.ID.NO. 27), and a portion of the FLAG epitope sequence. The ~630 bp product of this PCR reaction was gel purified and used as template in a subsequent PCR reaction employing the same reaction conditions but using primers BB47 and BB61 (5' CGCGAATTCTTATTTATCGTCATCGTCTTTGTAGTCCGAGCC TCC >3)(SEQ.ID.NO.30), which adds the remainder of the FLAG epitope sequence followed by a TAA stop codon and an *Eco* RI cloning site. The ~675 bp product of this PCR reaction was "cleaned up" using the QIAquick PCR Purification (Qiagen) according to the vendor protocol, digested with *Sty* I and *Eco* RI (New England BioLabs) according to the vendor protocols, ethanol-precipitated, resuspended in 20 µl of 10 mM Tris-HCl pH 8.5 and run out on a 2% agarose gel. The ~86 bp *Eco* RI - *Bsr* GI fragment of interest was gel purified using a QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol. This fragment was ligated with pBBT132 (described in Example 16) that had been cut with *Eco* RI and *Bsr* GI, treated with calf intestinal alkaline phosphatase (New England BioLabs) and gel purified.

The ligation reaction was used to transform *E. coli* and plasmids from resulting transformants were sequenced to identify a clone containing the *167C mutation and having the correct sequence throughout the ~86 bp *Eco* RI - *Bsr* GI segment.

For expression in baculovirus, the EPO genes encoding the 8 muteins were excised from the pUC19-based pBBT132 derivatives as *Bam* H I - *Eco* RI fragments of ~ 630 bp and subcloned into the pBlueBac4.5 baculovirus transfer vector used to express wild type EPO.

Using procedures similar to those described here, one can construct other cysteine muteins of EPO. The cysteine muteins can be substitution mutations that substitute cysteine for a natural amino residue in the EPO coding sequence, insertion mutations that insert a cysteine residue between two naturally occurring amino acids in the EPO coding sequence, or addition mutations that add a cysteine residue preceding the first amino acid, A1, of the EPO coding sequence or add a cysteine residue following the terminal amino acid residue, R166, of the EPO coding sequence. The cysteine residues can be substituted for any amino acid, or inserted between any two amino acids, anywhere in the EPO coding sequence. Preferred sites for substituting or inserting cysteine residues in EPO are in the region preceding Helix A, the A-B loop, the B-C loop, the C-D loop and the region distal to Helix D. Other preferred sites are the first or last three amino acids of the A, B, C and D Helices. Preferred residues in these regions for creating cysteine substitutions are A1, P2, P3, R4, L5, D8, S9, I25, T27, G28, A30, E31, H32, S34, N36, I39, D43, T44, K45, N47, Y49, A50, K52, R53, M54, E55, G57, Q58, G77, Q78, A79, S84, Q86, W88, E89, T107, R110, A111, G113, A114, Q115, K116, E117, A118, S120, P121, P122, D123, A124, A125, A127, A128, R131, T132, K154, Y156, T157, G158, E159, A160, T163, G164, D165, R166. Cysteine residues also can be inserted immediately preceding or following these amino acids. Another preferred site for adding a cysteine residue would be preceding A1, which we refer to as *-1C.

One also can construct EPO muteins containing a free cysteine by substituting another amino acid for one of the naturally-occurring cysteine residues in EPO. The naturally-occurring cysteine residue that normally forms a disulfide bond with the substituted cysteine residue is now free. The non-essential cysteine residue can be replaced with any of the other 19 amino acids, but preferably with a serine or alanine residue. A free cysteine residue also can be introduced into EPO by chemical modification of a naturally occurring amino acid using procedures such as those described by Sytkowski et al. (1998).

Using procedures similar to those described in Examples 16 – 19, one can express the proteins in eukaryotic cells (e.g., insect cells or mammalian cells), purify the proteins, PEGylate the proteins and measure their bioactivities in an *in vitro* bioassay. The EPO muteins also can be expressed in prokaryotic cells such as *E. coli* using procedures similar to those described in Examples 1-15, or related procedures well known to those skilled in the art.

B. Insect Cell Expression of EPO-Cys Muteins.

For expression experiments, the eight plasmids encoding mutant EPO genes were used to cotransfect Sf 9 cells along with linearized Bac-N-Blue™ DNA using the procedures described above

for plasmid pBBT138, which encodes wild type EPO. The transfection supernatants were assayed on Sf 9 cells for blue-plaque formation. For each mutant, ten blue plaques were picked and subcultured as described in Example 16. Five of the ten resulting supernatants were screened by SDS-PAGE and Western blot to detect expression of the EPO muteins. Western blot analysis was carried out using the procedure described in Example 16 for wild type EPO. Western results showed that at least 3 of the 5 supernatants screened from each clone contained FLAG-tagged EPO protein. Western blot results also revealed that plasmids encoding muteins that should prevent glycosylation at one of the N-linked glycosylation sites (N24C, T26C, N38C, T40C, N83C and S85C) yielded proteins with molecular weights approximately 2,200-2,800 daltons smaller than wild type BV rEPO. In contrast, the S126 mut⁵ 10 mut¹⁰ 15 mut¹⁵ 20 mut²⁰ 25 mut²⁵ 30 mut³⁰ 35 mut³⁵ 40 mut⁴⁰ 45 mut⁴⁵ 50 mut⁵⁰ 55 mut⁵⁵ 60 mut⁶⁰ 65 mut⁶⁵ 70 mut⁷⁰ 75 mut⁷⁵ 80 mut⁸⁰ 85 mut⁸⁵ 90 mut⁹⁰ 95 mut⁹⁵ 100 mut¹⁰⁰ 105 mut¹⁰⁵ 110 mut¹¹⁰ 115 mut¹¹⁵ 120 mut¹²⁰ 125 mut¹²⁵ 130 mut¹³⁰ 135 mut¹³⁵ 140 mut¹⁴⁰ 145 mut¹⁴⁵ 150 mut¹⁵⁰ 155 mut¹⁵⁵ 160 mut¹⁶⁰ 165 mut¹⁶⁵ 170 mut¹⁷⁰ 175 mut¹⁷⁵ 180 mut¹⁸⁰ 185 mut¹⁸⁵ 190 mut¹⁹⁰ 195 mut¹⁹⁵ 200 mut²⁰⁰ 205 mut²⁰⁵ 210 mut²¹⁰ 215 mut²¹⁵ 220 mut²²⁰ 225 mut²²⁵ 230 mut²³⁰ 235 mut²³⁵ 240 mut²⁴⁰ 245 mut²⁴⁵ 250 mut²⁵⁰ 255 mut²⁵⁵ 260 mut²⁶⁰ 265 mut²⁶⁵ 270 mut²⁷⁰ 275 mut²⁷⁵ 280 mut²⁸⁰ 285 mut²⁸⁵ 290 mut²⁹⁰ 295 mut²⁹⁵ 300 mut³⁰⁰ 305 mut³⁰⁵ 310 mut³¹⁰ 315 mut³¹⁵ 320 mut³²⁰ 325 mut³²⁵ 330 mut³³⁰ 335 mut³³⁵ 340 mut³⁴⁰ 345 mut³⁴⁵ 350 mut³⁵⁰ 355 mut³⁵⁵ 360 mut³⁶⁰ 365 mut³⁶⁵ 370 mut³⁷⁰ 375 mut³⁷⁵ 380 mut³⁸⁰ 385 mut³⁸⁵ 390 mut³⁹⁰ 395 mut³⁹⁵ 400 mut⁴⁰⁰ 405 mut⁴⁰⁵ 410 mut⁴¹⁰ 415 mut⁴¹⁵ 420 mut⁴²⁰ 425 mut⁴²⁵ 430 mut⁴³⁰ 435 mut⁴³⁵ 440 mut⁴⁴⁰ 445 mut⁴⁴⁵ 450 mut⁴⁵⁰ 455 mut⁴⁵⁵ 460 mut⁴⁶⁰ 465 mut⁴⁶⁵ 470 mut⁴⁷⁰ 475 mut⁴⁷⁵ 480 mut⁴⁸⁰ 485 mut⁴⁸⁵ 490 mut⁴⁹⁰ 495 mut⁴⁹⁵ 500 mut⁵⁰⁰ 505 mut⁵⁰⁵ 510 mut⁵¹⁰ 515 mut⁵¹⁵ 520 mut⁵²⁰ 525 mut⁵²⁵ 530 mut⁵³⁰ 535 mut⁵³⁵ 540 mut⁵⁴⁰ 545 mut⁵⁴⁵ 550 mut⁵⁵⁰ 555 mut⁵⁵⁵ 560 mut⁵⁶⁰ 565 mut⁵⁶⁵ 570 mut⁵⁷⁰ 575 mut⁵⁷⁵ 580 mut⁵⁸⁰ 585 mut⁵⁸⁵ 590 mut⁵⁹⁰ 595 mut⁵⁹⁵ 600 mut⁶⁰⁰ 605 mut⁶⁰⁵ 610 mut⁶¹⁰ 615 mut⁶¹⁵ 620 mut⁶²⁰ 625 mut⁶²⁵ 630 mut⁶³⁰ 635 mut⁶³⁵ 640 mut⁶⁴⁰ 645 mut⁶⁴⁵ 650 mut⁶⁵⁰ 655 mut⁶⁵⁵ 660 mut⁶⁶⁰ 665 mut⁶⁶⁵ 670 mut⁶⁷⁰ 675 mut⁶⁷⁵ 680 mut⁶⁸⁰ 685 mut⁶⁸⁵ 690 mut⁶⁹⁰ 695 mut⁶⁹⁵ 700 mut⁷⁰⁰ 705 mut⁷⁰⁵ 710 mut⁷¹⁰ 715 mut⁷¹⁵ 720 mut⁷²⁰ 725 mut⁷²⁵ 730 mut⁷³⁰ 735 mut⁷³⁵ 740 mut⁷⁴⁰ 745 mut⁷⁴⁵ 750 mut⁷⁵⁰ 755 mut⁷⁵⁵ 760 mut⁷⁶⁰ 765 mut⁷⁶⁵ 770 mut⁷⁷⁰ 775 mut⁷⁷⁵ 780 mut⁷⁸⁰ 785 mut⁷⁸⁵ 790 mut⁷⁹⁰ 795 mut⁷⁹⁵ 800 mut⁸⁰⁰ 805 mut⁸⁰⁵ 810 mut⁸¹⁰ 815 mut⁸¹⁵ 820 mut⁸²⁰ 825 mut⁸²⁵ 830 mut⁸³⁰ 835 mut⁸³⁵ 840 mut⁸⁴⁰ 845 mut⁸⁴⁵ 850 mut⁸⁵⁰ 855 mut⁸⁵⁵ 860 mut⁸⁶⁰ 865 mut⁸⁶⁵ 870 mut⁸⁷⁰ 875 mut⁸⁷⁵ 880 mut⁸⁸⁰ 885 mut⁸⁸⁵ 890 mut⁸⁹⁰ 895 mut⁸⁹⁵ 900 mut⁹⁰⁰ 905 mut⁹⁰⁵ 910 mut⁹¹⁰ 915 mut⁹¹⁵ 920 mut⁹²⁰ 925 mut⁹²⁵ 930 mut⁹³⁰ 935 mut⁹³⁵ 940 mut⁹⁴⁰ 945 mut⁹⁴⁵ 950 mut⁹⁵⁰ 955 mut⁹⁵⁵ 960 mut⁹⁶⁰ 965 mut⁹⁶⁵ 970 mut⁹⁷⁰ 975 mut⁹⁷⁵ 980 mut⁹⁸⁰ 985 mut⁹⁸⁵ 990 mut⁹⁹⁰ 995 mut⁹⁹⁵ 1000 mut¹⁰⁰⁰ 1005 mut¹⁰⁰⁵ 1010 mut¹⁰¹⁰ 1015 mut¹⁰¹⁵ 1020 mut¹⁰²⁰ 1025 mut¹⁰²⁵ 1030 mut¹⁰³⁰ 1035 mut¹⁰³⁵ 1040 mut¹⁰⁴⁰ 1045 mut¹⁰⁴⁵ 1050 mut¹⁰⁵⁰ 1055 mut¹⁰⁵⁵ 1060 mut¹⁰⁶⁰ 1065 mut¹⁰⁶⁵ 1070 mut¹⁰⁷⁰ 1075 mut¹⁰⁷⁵ 1080 mut¹⁰⁸⁰ 1085 mut¹⁰⁸⁵ 1090 mut¹⁰⁹⁰ 1095 mut¹⁰⁹⁵ 1100 mut¹¹⁰⁰ 1105 mut¹¹⁰⁵ 1110 mut¹¹¹⁰ 1115 mut¹¹¹⁵ 1120 mut¹¹²⁰ 1125 mut¹¹²⁵ 1130 mut¹¹³⁰ 1135 mut¹¹³⁵ 1140 mut¹¹⁴⁰ 1145 mut¹¹⁴⁵ 1150 mut¹¹⁵⁰ 1155 mut¹¹⁵⁵ 1160 mut¹¹⁶⁰ 1165 mut¹¹⁶⁵ 1170 mut¹¹⁷⁰ 1175 mut¹¹⁷⁵ 1180 mut¹¹⁸⁰ 1185 mut¹¹⁸⁵ 1190 mut¹¹⁹⁰ 1195 mut¹¹⁹⁵ 1200 mut¹²⁰⁰ 1205 mut¹²⁰⁵ 1210 mut¹²¹⁰ 1215 mut¹²¹⁵ 1220 mut¹²²⁰ 1225 mut¹²²⁵ 1230 mut¹²³⁰ 1235 mut¹²³⁵ 1240 mut¹²⁴⁰ 1245 mut¹²⁴⁵ 1250 mut¹²⁵⁰ 1255 mut¹²⁵⁵ 1260 mut¹²⁶⁰ 1265 mut¹²⁶⁵ 1270 mut¹²⁷⁰ 1275 mut¹²⁷⁵ 1280 mut¹²⁸⁰ 1285 mut¹²⁸⁵ 1290 mut¹²⁹⁰ 1295 mut¹²⁹⁵ 1300 mut¹³⁰⁰ 1305 mut¹³⁰⁵ 1310 mut¹³¹⁰ 1315 mut¹³¹⁵ 1320 mut¹³²⁰ 1325 mut¹³²⁵ 1330 mut¹³³⁰ 1335 mut¹³³⁵ 1340 mut¹³⁴⁰ 1345 mut¹³⁴⁵ 1350 mut¹³⁵⁰ 1355 mut¹³⁵⁵ 1360 mut¹³⁶⁰ 1365 mut¹³⁶⁵ 1370 mut¹³⁷⁰ 1375 mut¹³⁷⁵ 1380 mut¹³⁸⁰ 1385 mut¹³⁸⁵ 1390 mut¹³⁹⁰ 1395 mut¹³⁹⁵ 1400 mut¹⁴⁰⁰ 1405 mut¹⁴⁰⁵ 1410 mut¹⁴¹⁰ 1415 mut¹⁴¹⁵ 1420 mut¹⁴²⁰ 1425 mut¹⁴²⁵ 1430 mut¹⁴³⁰ 1435 mut¹⁴³⁵ 1440 mut¹⁴⁴⁰ 1445 mut¹⁴⁴⁵ 1450 mut¹⁴⁵⁰ 1455 mut¹⁴⁵⁵ 1460 mut¹⁴⁶⁰ 1465 mut¹⁴⁶⁵ 1470 mut¹⁴⁷⁰ 1475 mut¹⁴⁷⁵ 1480 mut¹⁴⁸⁰ 1485 mut¹⁴⁸⁵ 1490 mut¹⁴⁹⁰ 1495 mut¹⁴⁹⁵ 1500 mut¹⁵⁰⁰ 1505 mut¹⁵⁰⁵ 1510 mut¹⁵¹⁰ 1515 mut¹⁵¹⁵ 1520 mut¹⁵²⁰ 1525 mut¹⁵²⁵ 1530 mut¹⁵³⁰ 1535 mut¹⁵³⁵ 1540 mut¹⁵⁴⁰ 1545 mut¹⁵⁴⁵ 1550 mut¹⁵⁵⁰ 1555 mut¹⁵⁵⁵ 1560 mut¹⁵⁶⁰ 1565 mut¹⁵⁶⁵ 1570 mut¹⁵⁷⁰ 1575 mut¹⁵⁷⁵ 1580 mut¹⁵⁸⁰ 1585 mut¹⁵⁸⁵ 1590 mut¹⁵⁹⁰ 1595 mut¹⁵⁹⁵ 1600 mut¹⁶⁰⁰ 1605 mut¹⁶⁰⁵ 1610 mut¹⁶¹⁰ 1615 mut¹⁶¹⁵ 1620 mut¹⁶²⁰ 1625 mut¹⁶²⁵ 1630 mut¹⁶³⁰ 1635 mut¹⁶³⁵ 1640 mut¹⁶⁴⁰ 1645 mut¹⁶⁴⁵ 1650 mut¹⁶⁵⁰ 1655 mut¹⁶⁵⁵ 1660 mut¹⁶⁶⁰ 1665 mut¹⁶⁶⁵ 1670 mut¹⁶⁷⁰ 1675 mut¹⁶⁷⁵ 1680 mut¹⁶⁸⁰ 1685 mut¹⁶⁸⁵ 1690 mut¹⁶⁹⁰ 1695 mut¹⁶⁹⁵ 1700 mut¹⁷⁰⁰ 1705 mut¹⁷⁰⁵ 1710 mut¹⁷¹⁰ 1715 mut¹⁷¹⁵ 1720 mut¹⁷²⁰ 1725 mut¹⁷²⁵ 1730 mut¹⁷³⁰ 1735 mut¹⁷³⁵ 1740 mut¹⁷⁴⁰ 1745 mut¹⁷⁴⁵ 1750 mut¹⁷⁵⁰ 1755 mut¹⁷⁵⁵ 1760 mut¹⁷⁶⁰ 1765 mut¹⁷⁶⁵ 1770 mut¹⁷⁷⁰ 1775 mut¹⁷⁷⁵ 1780 mut¹⁷⁸⁰ 1785 mut¹⁷⁸⁵ 1790 mut¹⁷⁹⁰ 1795 mut¹⁷⁹⁵ 1800 mut¹⁸⁰⁰ 1805 mut¹⁸⁰⁵ 1810 mut¹⁸¹⁰ 1815 mut¹⁸¹⁵ 1820 mut¹⁸²⁰ 1825 mut¹⁸²⁵ 1830 mut¹⁸³⁰ 1835 mut¹⁸³⁵ 1840 mut¹⁸⁴⁰ 1845 mut¹⁸⁴⁵ 1850 mut¹⁸⁵⁰ 1855 mut¹⁸⁵⁵ 1860 mut¹⁸⁶⁰ 1865 mut¹⁸⁶⁵ 1870 mut¹⁸⁷⁰ 1875 mut¹⁸⁷⁵ 1880 mut¹⁸⁸⁰ 1885 mut¹⁸⁸⁵ 1890 mut¹⁸⁹⁰ 1895 mut¹⁸⁹⁵ 1900 mut¹⁹⁰⁰ 1905 mut¹⁹⁰⁵ 1910 mut¹⁹¹⁰ 1915 mut¹⁹¹⁵ 1920 mut¹⁹²⁰ 1925 mut¹⁹²⁵ 1930 mut¹⁹³⁰ 1935 mut¹⁹³⁵ 1940 mut¹⁹⁴⁰ 1945 mut¹⁹⁴⁵ 1950 mut¹⁹⁵⁰ 1955 mut¹⁹⁵⁵ 1960 mut¹⁹⁶⁰ 1965 mut¹⁹⁶⁵ 1970 mut¹⁹⁷⁰ 1975 mut¹⁹⁷⁵ 1980 mut¹⁹⁸⁰ 1985 mut¹⁹⁸⁵ 1990 mut¹⁹⁹⁰ 1995 mut¹⁹⁹⁵ 2000 mut²⁰⁰⁰ 2005 mut²⁰⁰⁵ 2010 mut²⁰¹⁰ 2015 mut²⁰¹⁵ 2020 mut²⁰²⁰ 2025 mut²⁰²⁵ 2030 mut²⁰³⁰ 2035 mut²⁰³⁵ 2040 mut²⁰⁴⁰ 2045 mut²⁰⁴⁵ 2050 mut²⁰⁵⁰ 2055 mut²⁰⁵⁵ 2060 mut²⁰⁶⁰ 2065 mut²⁰⁶⁵ 2070 mut²⁰⁷⁰ 2075 mut²⁰⁷⁵ 2080 mut²⁰⁸⁰ 2085 mut²⁰⁸⁵ 2090 mut²⁰⁹⁰ 2095 mut²⁰⁹⁵ 2100 mut²¹⁰⁰ 2105 mut²¹⁰⁵ 2110 mut²¹¹⁰ 2115 mut²¹¹⁵ 2120 mut²¹²⁰ 2125 mut²¹²⁵ 2130 mut²¹³⁰ 2135 mut²¹³⁵ 2140 mut²¹⁴⁰ 2145 mut²¹⁴⁵ 2150 mut²¹⁵⁰ 2155 mut²¹⁵⁵ 2160 mut²¹⁶⁰ 2165 mut²¹⁶⁵ 2170 mut²¹⁷⁰ 2175 mut²¹⁷⁵ 2180 mut²¹⁸⁰ 2185 mut²¹⁸⁵ 2190 mut²¹⁹⁰ 2195 mut²¹⁹⁵ 2200 mut²²⁰⁰ 2205 mut²²⁰⁵ 2210 mut²²¹⁰ 2215 mut²²¹⁵ 2220 mut²²²⁰ 2225 mut²²²⁵ 2230 mut²²³⁰ 2235 mut²²³⁵ 2240 mut²²⁴⁰ 2245 mut²²⁴⁵ 2250 mut²²⁵⁰ 2255 mut²²⁵⁵ 2260 mut²²⁶⁰ 2265 mut²²⁶⁵ 2270 mut²²⁷⁰ 2275 mut²²⁷⁵ 2280 mut²²⁸⁰ 2285 mut²²⁸⁵ 2290 mut²²⁹⁰ 2295 mut²²⁹⁵ 2300 mut²³⁰⁰ 2305 mut²³⁰⁵ 2310 mut²³¹⁰ 2315 mut²³¹⁵ 2320 mut²³²⁰ 2325 mut²³²⁵ 2330 mut²³³⁰ 2335 mut²³³⁵ 2340 mut²³⁴⁰ 2345 mut²³⁴⁵ 2350 mut²³⁵⁰ 2355 mut²³⁵⁵ 2360 mut²³⁶⁰ 2365 mut²³⁶⁵ 2370 mut²³⁷⁰ 2375 mut²³⁷⁵ 2380 mut²³⁸⁰ 2385 mut²³⁸⁵ 2390 mut²³⁹⁰ 2395 mut²³⁹⁵ 2400 mut²⁴⁰⁰ 2405 mut²⁴⁰⁵ 2410 mut²⁴¹⁰ 2415 mut²⁴¹⁵ 2420 mut²⁴²⁰ 2425 mut²⁴²⁵ 2430 mut²⁴³⁰ 2435 mut²⁴³⁵ 2440 mut²⁴⁴⁰ 2445 mut²⁴⁴⁵ 2450 mut²⁴⁵⁰ 2455 mut²⁴⁵⁵ 2460 mut²⁴⁶⁰ 2465 mut²⁴⁶⁵ 2470 mut²⁴⁷⁰ 2475 mut²⁴⁷⁵ 2480 mut²⁴⁸⁰ 2485 mut²⁴⁸⁵ 2490 mut²⁴⁹⁰ 2495 mut²⁴⁹⁵ 2500 mut²⁵⁰⁰ 2505 mut²⁵⁰⁵ 2510 mut²⁵¹⁰ 2515 mut²⁵¹⁵ 2520 mut²⁵²⁰ 2525 mut²⁵²⁵ 2530 mut²⁵³⁰ 2535 mut²⁵³⁵ 2540 mut²⁵⁴⁰ 2545 mut²⁵⁴⁵ 2550 mut²⁵⁵⁰ 2555 mut²⁵⁵⁵ 2560 mut²⁵⁶⁰ 2565 mut²⁵⁶⁵ 2570 mut²⁵⁷⁰ 2575 mut²⁵⁷⁵ 2580 mut²⁵⁸⁰ 2585 mut²⁵⁸⁵ 2590 mut²⁵⁹⁰ 2595 mut²⁵⁹⁵ 2600 mut²⁶⁰⁰ 2605 mut²⁶⁰⁵ 2610 mut²⁶¹⁰ 2615 mut²⁶¹⁵ 2620 mut²⁶²⁰ 2625 mut²⁶²⁵ 2630 mut²⁶³⁰ 2635 mut²⁶³⁵ 2640 mut²⁶⁴⁰ 2645 mut²⁶⁴⁵ 2650 mut²⁶⁵⁰ 2655 mut²⁶⁵⁵ 2660 mut²⁶⁶⁰ 2665 mut²⁶⁶⁵ 2670 mut²⁶⁷⁰ 2675 mut²⁶⁷⁵ 2680 mut²⁶⁸⁰ 2685 mut²⁶⁸⁵ 2690 mut²⁶⁹⁰ 2695 mut²⁶⁹⁵ 2700 mut²⁷⁰⁰ 2705 mut²⁷⁰⁵ 2710 mut²⁷¹⁰ 2715 mut²⁷¹⁵ 2720 mut²⁷²⁰ 2725 mut²⁷²⁵ 2730 mut²⁷³⁰ 2735 mut²⁷³⁵ 2740 mut²⁷⁴⁰ 2745 mut²⁷⁴⁵ 2750 mut²⁷⁵⁰ 2755 mut²⁷⁵⁵ 2760 mut²⁷⁶⁰ 2765 mut²⁷⁶⁵ 2770 mut²⁷⁷⁰ 2775 mut²⁷⁷⁵ 2780 mut²⁷⁸⁰ 2785 mut²⁷⁸⁵ 2790 mut²⁷⁹⁰ 2795 mut²⁷⁹⁵ 2800 mut²⁸⁰⁰ 2805 mut²⁸⁰⁵ 2810 mut²⁸¹⁰ 2815 mut²⁸¹⁵ 2820 mut²⁸²⁰ 2825 mut²⁸²⁵ 2830 mut²⁸³⁰ 2835 mut²⁸³⁵ 2840 mut²⁸⁴⁰ 2845 mut²⁸⁴⁵ 2850 mut²⁸⁵⁰ 2855 mut²⁸⁵⁵ 2860 mut²⁸⁶⁰ 2865 mut²⁸⁶⁵ 2870 mut²⁸⁷⁰ 2875 mut²⁸⁷⁵ 2880 mut²⁸⁸⁰ 2885 mut²⁸⁸⁵ 2890 mut²⁸⁹⁰ 2895 mut²⁸⁹⁵ 2900 mut²⁹⁰⁰ 2905 mut²⁹⁰⁵ 2910 mut²⁹¹⁰ 2915 mut²⁹¹⁵ 2920 mut²⁹²⁰ 2925 mut²⁹²⁵ 2930 mut²⁹³⁰ 2935 mut²⁹³⁵ 2940 mut²⁹⁴⁰ 2945 mut²⁹⁴⁵ 2950 mut²⁹⁵⁰ 2955 mut²⁹⁵⁵ 2960 mut²⁹⁶⁰ 2965 mut²⁹⁶⁵ 2970 mut²⁹⁷⁰ 2975 mut²⁹⁷⁵ 2980 mut²⁹⁸⁰ 2985 mut²⁹⁸⁵ 2990 mut²⁹⁹⁰ 2995 mut²⁹⁹⁵ 3000 mut³⁰⁰⁰ 3005 mut³⁰⁰⁵ 3010 mut³⁰¹⁰ 3015 mut³⁰¹⁵ 3020 mut³⁰²⁰ 3025 mut³⁰²⁵ 3030 mut³⁰³⁰ 3035 mut³⁰³⁵ 3040 mut³⁰⁴⁰ 3045 mut³⁰⁴⁵ 3050 mut³⁰⁵⁰ 3055 mut³⁰⁵⁵ 3060 mut³⁰⁶⁰ 3065 mut³⁰⁶⁵ 3070 mut³⁰⁷⁰ 3075 mut³⁰⁷⁵ 3080 mut³⁰⁸⁰ 3085 mut³⁰⁸⁵ 3090 mut³⁰⁹⁰ 3095 mut³⁰⁹⁵ 3100 mut³¹⁰⁰ 3105 mut³¹⁰⁵ 3110 mut³¹¹⁰ 3115 mut³¹¹⁵ 3120 mut³¹²⁰ 3125 mut³¹²⁵ 3130 mut³¹³⁰ 3135 mut³¹³⁵ 3140 mut³¹⁴⁰ 3145 mut³¹⁴⁵ 3150 mut³¹⁵⁰ 3155 mut³¹⁵⁵ 3160 mut³¹⁶⁰ 3165 mut³¹⁶⁵ 3170 mut³¹⁷⁰ 3175 mut³¹⁷⁵ 3180 mut³¹⁸⁰ 3185 mut³¹⁸⁵ 3190 mut³¹⁹⁰ 3195 mut³¹⁹⁵ 3200 mut³²⁰⁰ 3205 mut³²⁰⁵ 3210 mut³²¹⁰ 3215 mut³²¹⁵ 3220 mut³²²⁰ 3225 mut³²²⁵ 3230 mut³²³⁰ 3235 mut³²³⁵ 3240 mut³²⁴⁰ 3245 mut³²⁴⁵ 3250 mut³²⁵⁰ 3255 mut³²⁵⁵ 3260 mut³²⁶⁰ 3265 mut³²⁶⁵ 3270 mut³²⁷⁰ 3275 mut³²⁷⁵ 3280 mut³²⁸⁰ 3285 mut³²⁸⁵ 3290 mut³²⁹⁰ 3295 mut³²⁹⁵ 3300 mut³³⁰⁰ 3305 mut³³⁰⁵ 3310 mut³³¹⁰ 3315 mut³³¹⁵ 3320 mut³³²⁰ 3325 mut³³²⁵ 3330 mut³³³⁰ 3335 mut³³³⁵ 3340 mut³³⁴⁰ 3345 mut³³⁴⁵ 3350 mut³³⁵⁰ 3355 mut³³⁵⁵ 3360 mut³³⁶⁰ 3365 mut³³⁶⁵ 3370 mut³³⁷⁰ 3375 mut³³⁷⁵ 3380 mut³³⁸⁰ 3385 mut³³⁸⁵ 3390 mut³³⁹⁰ 3395 mut³³⁹⁵ 3400 mut³⁴⁰⁰ 3405 mut³⁴⁰⁵ 3410 mut³⁴¹⁰ 3415 mut³⁴¹⁵ 3420 mut³⁴²⁰ 3425 mut³⁴²⁵ 3430 mut³⁴³⁰ 3435 mut³⁴³⁵ 3440 mut³⁴⁴⁰ 3445 mut³⁴⁴⁵ 3450 mut³⁴⁵⁰ 3455 mut³⁴⁵⁵ 3460 mut³⁴⁶⁰ 3465 mut³⁴⁶⁵ 3470 mut³⁴⁷⁰ 3475 mut³⁴⁷⁵ 3480 mut³⁴⁸⁰ 3485 mut³⁴⁸⁵ 3490 mut³⁴⁹⁰ 3495 mut³⁴⁹⁵ 3500 mut³⁵⁰⁰ 3505 mut³⁵⁰⁵ 3510 mut³⁵¹⁰ 3515 mut³⁵¹⁵ 3520 mut³⁵²⁰ 3525 mut³⁵²⁵ 3530 mut³⁵³⁰ 3535 mut³⁵³⁵ 3540 mut³⁵⁴⁰ 3545 mut³⁵⁴⁵ 3550 mut³⁵⁵⁰ 3555 mut³⁵⁵⁵ 3560 mut³⁵⁶⁰ 3565 mut³⁵⁶⁵ 3570 mut³⁵⁷⁰ 3575 mut³⁵⁷⁵ 3580 mut³⁵⁸⁰ 3585 mut³⁵⁸⁵ 3590 mut³⁵⁹⁰ 3595 mut³⁵⁹⁵ 3600 mut³⁶⁰⁰ 3605 mut³⁶⁰⁵ 3610 mut³⁶¹⁰ 3615 mut³⁶¹⁵ 3620 mut³⁶²⁰ 3625 mut³⁶²⁵ 3630 mut³⁶³⁰ 3635 mut³⁶³⁵ 3640 mut³⁶⁴⁰ 3645 mut³⁶⁴⁵ 3650 mut³⁶⁵⁰ 3655 mut³⁶⁵⁵ 3660 mut³⁶⁶⁰ 3665 mut³⁶⁶⁵ 3670 mut³⁶⁷⁰ 3675 mut³⁶⁷⁵ 3680 mut³⁶⁸⁰ 3685 mut³⁶⁸⁵ 3690 mut³⁶⁹⁰ 3695 mut³⁶⁹⁵ 3700 mut³⁷⁰⁰ 3705 mut³⁷⁰⁵ 3710 mut³⁷¹⁰ 3715 mut³⁷¹⁵ 3720 mut³⁷²⁰ 3725 mut³⁷²⁵ 3730 mut³⁷³⁰ 3735 mut³⁷³⁵ 3740 mut³⁷⁴⁰ 3745 mut³⁷⁴⁵ 3750 mut³⁷⁵⁰ 3755 mut³⁷⁵⁵ 3760 mut³⁷⁶⁰ 3765 mut³⁷⁶⁵ 3770 mut³⁷⁷⁰ 3775 mut³⁷⁷⁵ 3780 mut³⁷⁸⁰ 3785 mut³⁷⁸⁵ 3790 mut³⁷⁹⁰ 3795 mut³⁷⁹⁵ 3800 mut³⁸⁰⁰ 3805 mut³⁸⁰⁵ 3810 mut³⁸¹⁰ 3815 mut³⁸¹⁵ 3820 mut³⁸²⁰ 3825 mut³⁸²⁵ 3830 mut³⁸³⁰ 3835 mut³⁸³⁵ 3840 mut³⁸⁴⁰ 3845 mut³⁸⁴⁵ 3850 mut³⁸⁵⁰ 3855 mut³⁸⁵⁵ 3860 mut³⁸⁶⁰ 3865 mut³⁸⁶⁵ 3870 mut³⁸⁷⁰ 3875 mut³⁸⁷⁵ 3880 mut³⁸⁸⁰ 3885 mut³⁸⁸⁵ 3890 mut³⁸⁹⁰ 3895 mut³⁸⁹⁵ 3900 mut³⁹⁰⁰ 3905

slightly smaller than wild type BV rEPO. Surprisingly, the S85C protein migrated as a doublet under both reducing and non-reducing conditions; the major band of the doublet was the expected size for a protein lacking glycosylation at one of the N-linked glycosylation sites, while the minor band was the size expected for fully glycosylated wild-type BV rEPO. All of the original recombinant Baculovirus
5 plaques isolated for the S85C mutein yielded rEPO proteins that migrated as doublets, suggesting that the doublet is due to partial glycosylation at N83 (or another amino acid) rather than contamination of S85C with wild type BV EPO or S126C or *167C proteins. Several of the muteins, e.g., S126C, *167C, had small amounts of a second protein that migrated with an apparent molecular weight of 45-55 kDa, depending upon the mutein. This is the molecular weight expected for disulfide-linked
10 rEPO dimers. These bands reacted with the anti-FLAG antibody in Western blots and were absent when the SDS gels were run under reducing conditions. These data indicate that the 45-55 kDa bands represent disulfide-linked rEPO-Cys dimers.

C. Bioactivities of EPO Cysteine Muteins.

15 Biological activities of the purified rEPO- Cys muteins were measured in the UT7/epo cell proliferation assay described in Example 16. Protein concentrations were determined using a Bradford assay. Because of the large number of samples the muteins were divided into two groups for analysis: muteins T26C, T40C, N83C and S126C comprised one group and muteins N24C, N38C, S85C and *167C comprised the second group. Muteins within a group were analyzed on the same
20 day. Wild type BV rEPO and CHO rEPO were analyzed in parallel on the same days to control for interday variability in the assays. All of the muteins stimulated proliferation of UT7/epo cells and had EC₅₀s that were within three-fold of the wild type rEPO control proteins. Mean EC₅₀s for the N24C, T26C, N83C, S85C, S126 and *167C muteins were similar to the mean EC₅₀s for wild type BV rEPO and CHO rEPO, averaging 0.3-0.8 ng/ml (Table 2). Mean EC₅₀s for the N38C and T40C proteins
25 were approximately 1 ng/ml (Table 2).

Bill et al., (1995) reported expression of the EPO cysteine muteins, N24C, N38C and N83C, in *E. coli*. In contrast to our results, they reported that bioactivities of these cysteine muteins were significantly reduced relative to wild type EPO. Bioactivity of the N38C mutein was reduced to less than 20% of wild type EPO bioactivity and bioactivities of the N24C and N83C muteins were reduced
30 to less than 5% of wild type EPO bioactivity. EC₅₀s were not reported. Bill et al. (1995) postulated that the reduced activities of the cysteine muteins were due to improper folding due to incorrect formation of disulfide bridges resulting from the extra cysteine residues introduced into the proteins. They did not employ cystine or other cysteine-blocking agents in the solutions used to purify the cysteine muteins.

35 Based upon the results of Bill et al. (1995), it was surprising that our purified N24C, N38C and N83C muteins had biological activities equal to, or within 3-fold of, wild type EPO. The data indicate that our methods for expression and purification of the EPO cysteine muteins results in proteins with superior *in vitro* biological activities compared to the methods employed by Bill et al. (1995).

Table 2. Properties of Human EPO Cysteine Muteins

Expression Plasmid	EPO Protein	Protein Recovery $\mu\text{g} / 500 \text{ ml}$	Mutation Location	Mean EC_{50} (ng/ml)	EC_{50} Range ¹
-	rEPO (CHO)	-	-	0.50	0.29–0.65 (N=6)
pBBT138	rEPO (BV)	345	-	0.37	0.21–0.51 (N=6)
pBBT150	N24C	75	A-B loop	0.76	0.58, 0.85, 0.85
pBBT151	T26C	805	A-B loop	0.27	0.21, 0.22, 0.39
pBBT152	N38C	85	A-B loop	1.05	0.65, 1.20, 1.30
pBBT161	T40C	102	A-B loop	0.95	0.60, 0.75, 1.50
pBBT162	N83C	96	B-C loop	0.50	0.42, 0.49, 0.60
pBBT153	S85C	135	B-C loop	0.72	0.50, 0.80, 0.85
pBBT154	S126C	220	C-D loop	0.31	0.25, 0.25, 0.42
pBBT155	*167C	129	C-terminus	0.61	0.51, 0.65, 0.68

¹ Data from individual experiments. A range is shown when $N > 3$.

5

EXAMPLE 18**PEGylation of EPO Cysteine Muteins****A. Small-Scale PEGylation of EPO Cysteine Muteins**

Several cysteine muteins and wild type EPO were tested for their ability to be PEGylated with a 5 kDa cysteine-reactive PEG following treatment with the reducing agent TCEP (Tris (2-carboxyethyl) phosphine-HCl). A titration study was performed with the T26C mutein to identify appropriate TCEP and PEG reagent concentrations for PEGylation, while avoiding over-reduction of the protein. We monitored partial reduction of the protein by SDS-PAGE, using wild type BV rEPO as a control. Increasing concentrations of TCEP (0.5 M to 6.0 M excess) in the presence of a 10–40-fold molar excess of either vinylsulfone or maleimide 5kDa PEG (Fluka) were tested. The reactions were analyzed by non-reducing SDS-PAGE. Wild type BV rEPO was treated in parallel as a control to identify partial reduction conditions that yielded significant amounts of monoPEGylated EPO-Cys muteins (a single PEG molecule attached to the EPO-Cys mutein), but no PEGylation of wild type BV rEPO. A 5-fold molar excess of TCEP and a 30-fold molar excess of 5 kDa vinylsulfone-PEG yielded significant amounts of monoPEGylated T26C protein without PEGylation of wild type BV rEPO.

Based on our findings with the T26C mutein, the following conditions were used to PEGylate several other cysteine muteins. Aliquots (1–2 μg) of purified BV rEPO and EPO-Cys muteins were incubated for 1 hr with a 5X molar excess of TCEP and a 30X molar excess of 5 kDa vinylsulfone PEG at pH 8.0 at room temperature. The reactions were stopped by dilution into SDS sample buffer (without reducing reagent) and analyzed by SDS-PAGE. Four cysteine muteins (N24C, T26C, S126C and *167C) were readily monoPEGylated under these conditions, as evidenced by the appearance of a new protein band migrating at approximately 35 kDa. The 35 kDa species is the size expected for mono-PEGylated EPO; no diPEGylated species, which are expected to have molecular weights of approximately 40 kDa, were detected for any of the EPO-cys muteins under these reaction conditions. Control experiments indicated that the cysteine muteins needed to be reduced with TCEP in order to be PEGylated. Wild type BV EPO did not PEGylate under identical partial reducing conditions.

These data indicate that the PEG molecule is attached to the free cysteine introduced into the N24C, T26C, S126C and *167C EPO-Cys muteins.

B. Preparation of PEG-T26C and PEG-S126C for Bioactivity Measurements.

5 We PEGylated larger quantities of the T26C and S126C proteins so that the PEGylated proteins could be purified for bioactivity measurements. PEGylation reactions were scaled to include 75 µg of each protein and the same molar ratios of TCEP and 5kDa-PEG used in the smaller 1 µg reactions. After 1 hour, the PEGylation mixture was diluted 10X with 20 mM Tris-HCl, pH 8.0, 20% glycerol (Buffer A) and loaded immediately onto a 1 ml Q-Sepharose column equilibrated in
10 Buffer A. The column was washed with equilibration buffer and bound proteins eluted with a linear 10 volume increasing salt gradient from 0 to 150 mM NaCl in 20 mM Tris-HCl, pH 8.0, 20% glycerol. The presence of the PEG moiety decreased the protein's affinity for the resin, allowing the PEGylated protein to be separated from the non-PEGylated protein. Fractions containing mono-PEGylated protein were identified by SDS-PAGE, followed by Coomassie Blue staining. Fractions
15 containing PEG-T26C but no visible underivatized protein, were stored frozen at -80°C and subsequently used in bioassays. Similar procedures were used to obtain purified PEG-S126C. A Western blot was performed to assess the purity of the PEG-T26C and PEG-S126C preparations used in bioassays. The Western blot was performed as described in Example 16. The Western blot gave strong signals at the sizes expected for PEG-T26C and PEG-S126C, but failed to detect any protein
20 migrating at the positions expected for the unPEGylated S126C and T26C proteins. From these results, we conclude there is little (<5 %) unPEGylated protein present in the purified PEGylated muteins.

C. Bioactivities of PEG-T26C and PEG-S126C Cysteine Muteins

25 The biological activities of the purified PEG-T26C and PEG-S126C proteins were measured in the UT7/epo cell proliferation assay described in Example 16. Protein concentrations of the PEG-EPO-Cys muteins were quantitated using a human EPO ELISA kit (R & D Systems), following the manufacturer's suggested directions. Protein concentrations of the non-PEGylated muteins and wild type BV rEPO also were quantitated by ELISA. The ELISA assay was performed on all the proteins
30 on the same day. Serial three-fold dilutions of the protein samples were prepared and analyzed in the bioassay. Unused material from each serial dilution was stored frozen at -80°C and later analyzed in the ELISA to determine an accurate protein concentration for the starting material. Several of the serial dilutions for each protein sample were analyzed to ensure that the protein concentration in at least one test sample fell within the linear range of the standard ELISA curve, which is 0.0025 – 0.2
35 Units/ml or approximately 0.02 – 1.6 ng/ml. At least two of the serial dilutions for each sample fell within this linear range.

Biological activities for the PEG-T26C and PEG-S126C muteins were similar to those of the non-modified T26C and S126C proteins and wild type BV rEPO. Mean EC₅₀s for the PEG-T26C and PEG-S126C muteins were similar to the mean EC₅₀ values determined for the non-PEGylated T26C

and S126C muteins and wild type BV rEPO, ranging from 0.48 – 0.82 ng/ml (Table 3). Biologically active, PEGylated EPO proteins have not been described previously.

Table 3. Bioactivities of PEG-Cys EPO Muteins

EPO Protein	Mean EC ₅₀ (ng/ml)	EC ₅₀ Range ¹ (ng/ml)
BV EPO	0.82	0.55, 0.95, 0.95
T26C	0.73	0.50, 0.85, 0.85
PEG-T26C	0.58	0.35, 0.65, 0.75
S126C	0.74	0.65, 0.75, 0.82
PEG-S126C	0.48	0.38, 0.52, 0.55

¹ Data from three experiments.

Example 19

Expression and purification of EPO cysteine muteins in mammalian cells.

The EPO cysteine muteins also can be expressed in mammalian cells and purified from the conditioned media. The EPO cysteine muteins can be expressed by transient transfection of mammalian cells or by constructing stably transformed mammalian cell lines expressing the EPO cysteine muteins. For therapeutic applications in humans, it is preferable that the EPO cysteine muteins be expressed without the linker and FLAG sequences. Monkey COS cells (available from the American Type culture Collection) can be used for transient transfection experiments, using procedures well known to those of skill in the art. A number of commercial sources, e.g., GIBCO/BRL sell lipid transfection reagents and provide detailed protocols that can be used to express the EPO cysteine muteins by transient transfection. Wild type EPO is manufactured for use in humans using stably transformed Chinese hamster ovary (CHO) cells expressing the protein. Stably transfected CHO cell lines are widely used for high-level expression of recombinant proteins (Geisse et al. 1996; Trill et al. 1995). In CHO cells, high level expression of chromosomally integrated heterologous genes can be achieved by gene amplification. Typically the gene of interest is linked to a marker gene for which amplification is selectable. A variety of genes which provide selections for amplification have been described (Kaufman 1990) but murine dihydrofolate reductase (dhfr) is frequently employed. Amplification of this gene confers resistance to the folate analog methotrexate (MTX) and the level of resistance increases with dhfr gene copy number (Alt et al., 1978). Utility of MTX selection is enhanced by the availability of CHO cell lines that are deficient in dhfr (Urlaub and Chasin, 1980).

One skilled in the art can construct expression vectors for the EPO cysteine muteins that incorporate the murine dhfr gene into the commercially available pCDNA3.1 expression vector (Invitrogen), which includes the neomycin phosphotransferase (NPT) gene that confers resistance to G418. The murine dhfr expression vector pdhfr2.9 is available from the American Type Culture Collection (catalogue No.37165). This dhfr gene is selectable in dhfr⁻ CHO cell lines and can be amplified by standard selections for MTX resistance (Crouse et al 1983). The dhfr coding sequence can be excised from pdhfr2.9 as a ~ 900 bp *Bgl* II fragment and cloned into the unique *Bam* HI site of the polylinker of the expression vector pREP4 (Invitrogen). This construct will position the gene

downstream of the strong RSV promoter, which is known to function in CHO cells (Trill et al. 1995) and upstream of a polyadenylation site derived from SV40. This dhfr expression cassette can then be excised from pREP4 as a *Sal* I fragment since *Sal* I sites closely flank the promoter and polyA addition site. Using oligonucleotide linkers this *Sal* I fragment can be cloned into the unique *Bgl* II site of pCDNA3.1.

For expression in mammalian cells such as COS or CHO cells, the EPO cysteine mutein cDNAs can be modified by PCR-based mutagenesis. At the 5' end one can add a Kozak consensus sequence prior to the translational initiator ATG codon in order to enhance translation in mammalian cells. At the 3' end, the linker and FLAG sequences can be removed and the natural coding sequence and translation termination signal restored. These modified EPO cDNAs can then be cloned as *Bam* HI – *Eco* RI fragments into the polylinker of pCDNA3.1 for transient transfection experiments or into the polylinker of the the pCDNA3.1::dhfr vector for stable expression in mammalian cells. For mammalian cell expression, the mutant EPO genes encoding the cysteine muteins would be modified at the 5' end to incorporate a Kozak consensus sequence (GCCACC) immediately preceding the translational initiator ATG codon in order to enhance translation in mammalian cells. At the 3' end the linker and FLAG sequences would be removed and the natural coding sequence restored. These modifications could be accomplished by a variety of mutagenesis techniques that are well known to those skilled in the art. PCR-based mutagenesis procedures based on those described in Examples 4 and 17 could be employed. PCR primers BB302 (5' > CGCGGATCCGCCACCATGGGGGTGCAC GAATGTCCT >3)(SEQ.ID.NO.47) and BB303 (5' > CGCGAATTCTCATCTGTCCCCTGTCCTGCAGCC >3)(SEQ.ID.NO. 48) could be used to PCR the mutated EPO genes cloned in pUC19 or pBlueBac4.5 as templates. Forward primer BB302 anneals to the EPO gene sequence encoding the first seven amino acids of the EPO secretion signal, adds a Kozak consensus sequence, GCCACC, immediately preceding the translational initiator ATG codon, and includes a *Bam* HI site for cloning purposes. The reverse primer BB303 anneals to sequences encoding the carboxyterminal seven amino acids of the EPO coding sequence, adds a TGA translational stop codon following the EPO coding sequence and includes a *Eco* RI site for cloning purposes. The products of the individual PCR reactions using these primers and any mutant EPO gene template that has a mutation between these two primers, e.g. amino acid substitutions at amino acids 1 through 159 of the mature EPO coding sequence, will produce PCR products that can be purified, digested with *Bam* HI and *Eco* RI and cloned into a vector suitable for expression in mammalian cells such as pCDNA3.1(+) (Invitrogen). PCR primers 302 and 303 also could be used to modify EPO cysteine muteins in which a cysteine residue is added preceding the first amino acid, A1, of the mature EPO coding sequence, but distal to the EPO signal sequence. Mutations in the EPO sequence that encode the carboxyterminal seven amino acids, or the *167C mutation described in Example 17, would require the use of alternative reverse primers in place of BB303. These primers would be individually designed to include the mutated cysteine codon, at least 21 nucleotides at the 3' end of the oligo that exactly match the template target, and the *Eco* RI site for cloning purposes. For example the reverse primer BB304 (5' > CGCGAATTCTCAACATCT GTCCCCTGTCCTG CAGCC

>3)(SEQ.ID.NO. 49) and BB302 could be used in a PCR reaction with the mutated EPO *167C gene cloned in pUC19 or pBlueBac4.5 as template to generate a modified mutant EPO gene encoding the *167C mutein that would be suitable for expression in mammalian cell expression systems.

Endotoxin free plasmid DNAs are preferred for transfecting mammalian cells such as COS or dhfr⁻ CHO cells. Dhfr⁻ CHO cell lines can be obtained from a number of sources such as Dr. L. Chasin at Columbia University (CHO K1 DUKX B11) or from the American Type Culture Collection (CHO duk⁻, ATCC No. CRL-9096). The cells can be cultured in F12/DMEM medium supplemented with 10% FBS, glutamine, glycine, hypoxanthine, and thymidine (Lucas et al., 1996). Transfections can be carried out by electroporation or by using transfection reagents well known to those of skill in the art such as LipofectAMINE (Gibco BRL), using the vendor protocols and / or those described in the literature (Kaufman, 1990). One can select for dhfr⁺ transfectants in F12/DMEM supplemented with 7% dialyzed FCS and lacking glutamine, glycine, hypoxanthine, and thymidine (Lucas et al., 1996). Alternatively one can select for G418 resistance (encoded by the NPT gene of pCDNA3.1) and subsequently screen transfectants for the dhfr⁺ phenotype. Dhfr⁺ clones can be expanded in selection medium and culture supernatants screened for EPO cysteine mutein production using commercially available EPO ELISA kits (available from R & D Systems) or by Western blot using anti-EPO antisera (available from R&D Systems). Clones expressing the EPO cysteine mutein can then be pooled and subjected to multiple rounds of selection for MTX resistance at increasing drug concentration as described by Kaufman (1990). After each round of MTX selection, individual clones can be tested for EPO cysteine mutein production. These procedures are well described in the literature and have been used to express a variety of heterologous protein in CHO cells (reviewed in Kaufman, 1990).

Preferably, the media used to grow the CHO cells expressing the EPO cysteine muteins should contain cystine or another cysteine blocking agent. The EPO cysteine muteins can be purified from the conditioned medium of CHO cells using protocols similar to those described by Imai et al. (1990). After removal of the CHO cells by centrifugation, the EPO cysteine muteins can be purified from the supernatant by column chromatography using techniques well known to those of skill in the art. The column chromatography steps employed for the purification of the EPO cysteine muteins could include Blue Sepharose, hydroxyapatite, reversed phase, hydrophobic interaction, size exclusion and ion-exchange chromatography.

EXAMPLE 20

Cloning, Expression and Purification of IFN- α 2.

A. Cloning DNA sequences encoding IFN- α 2.

There are at least 25 distinct IFN- α genes which encode proteins that share 70% or greater amino acid identity (Blatt et al., 1996). Due to the high degree of DNA sequence homology between IFN- α species, the IFN- α 2 gene was cloned in two steps. First, the IFN- α 2 gene was amplified by PCR from human genomic DNA using primers corresponding to unique sequences upstream and downstream of the IFN- α 2 gene. This PCR product was cloned and sequenced to confirm that it encodes the IFN- α 2

gene. Subsequently, the IFN- α 2 coding sequence was modified by PCR and subcloned for expression in *E. coli* and site-directed mutagenesis. DNA encoding IFN- α 2 was amplified by PCR from human genomic DNA (CLONTECH). PCR reactions were carried out with BB93

(5>CGCGAATTCGGATATGTAAA TAGATACACAGTG>3) (SEQ.ID.NO. 50) and BB94

- 5 (5>CGCAAGCTTAAAAGATTTAAATCGTGTTCATGGT>3) (SEQ.ID.NO.51). BB93 anneals to genomic sequences ~300 bp upstream (i.e. 5' to) of the IFN- α 2 coding sequence and contains an *Eco* RI site for cloning purposes. BB94 anneals to genomic sequences ~100 bp downstream (i.e. 3' to) of the IFN- α 2 coding sequence and contains a *Hind* III site for cloning purposes. The resulting ~1 kb PCR product was digested with *Eco* RI and *Hind* III and cloned into similarly digested, and alkaline phosphatased, pCDNA3.1(+) (Invitrogen). A clone having the correct DNA sequence for IFN- α 2
- 10 (Henco et al, 1985) was identified and designated pBBT160.

- For cytoplasmic expression in *E. coli* the cloned IFN- α 2 gene of pBBT160 was modified by PCR to incorporate a methionine codon immediately prior to the first residue (C1) of the mature IFN- α 2 protein. A TAA stop codon was added following the carboxy-terminal residue, E165. At the same
- 15 time, *Xba* I and *Sal* I sites were added and a *Bgl* II site was eliminated in order to provide convenient restriction sites for subsequent mutagenesis. In this reaction pBBT160 was used as template and amplified by primers BB99 5>CGCAAGCTTCATATGTGTGATCTGCCTCAAACCCACAGCCTG GGTTCCTAGAAGGACCTTGATGCTC>3) (SEQ.ID.NO. 52) and BB100 (5>CGCGAATTCCTTATT CCTTACTTCTTAACTTTCTTGCAAGTTTGTGCAAAAGAAAAGGATCTCATGAT>3)
- 20 (SEQ.ID.NO. 53). BB99 anneals to the 5' end of the coding sequence of mature IFN- α 2 and encodes an initiator methionine preceding the first amino acid of mature IFN- α 2. BB99 introduces an *Xba* I site ~30 bp downstream of the initiator ATG, but the amino acid sequence of the protein is unaltered. A *Hind* III site and an *Nde* I site, which overlaps the ATG, were included for cloning purposes. The reverse primer, BB100, anneals to the 3' end of the coding sequence and adds a TAA stop codon.
- 25 BB100 introduces a *Sal* I site ~30 bp upstream of the TAA codon and eliminates a naturally occurring *Bgl* II site located ~15 bp further upstream. As a result, the naturally occurring *Bgl* II site located ~185 bp downstream of the initiator ATG becomes a unique site. None of these alterations changed the amino acid sequence. An *Eco* RI site was added immediately downstream of the stop codon for cloning purposes. The ~520 bp PCR product was digested with *Hind* III and *Eco* RI, gel purified and
- 30 cloned into similarly digested plasmid pCDNA3.1(+). One clone was determined to have the correct DNA sequence. This plasmid was designated pBBT164. For cytoplasmic expression in *E. coli*, the ~520 bp *Nde* I - *Eco* RI fragment of pBBT164 was cloned into similarly digested expression vector, pCYB1, (New England Biolabs). The plasmid vector pCYB1 allows genes to be expressed as unfused proteins or as fusion proteins; this construct was created so that the protein is expressed as an
- 35 unfused protein. The resulting plasmid was termed pBBT170 and encodes met-IFN- α 2. The *Nde* I-*Eco* RI fragment of pBBT164 containing the met-IFN- α 2 sequence also was subcloned into *Nde* I-*Eco* RI-digested pUC18 to generate plasmid pBBT168.

IFN- α 2 also can be expressed in an active form in *E. coli* by secretion into the periplasmic space (Voss et al., 1994). Secreted IFN- α 2 lacks an N-terminal methionine and has an amino acid

sequence identical to naturally occurring IFN- α 2. In order to express a secreted form of IFN- α 2, the leader sequence of the *E. coli* heat-stable enterotoxin (STII) gene (Picken et al., 1983) was fused to the coding sequence for mature IFN- α 2 via PCR. Because of its length, the STII sequence was added in two sequential PCR reactions. The first reaction used forward primer BB101 (5'-GCATCTATGTT
 5 CGTTTCTCTATCGCTACCAACGCTTACGCATGTGATCT GCCTCAAACCCAC AGC>3)
 (SEQ.ID.NO. 54) and reverse primer BB100 with pBBT164 (described above) as template. The 3' end (21 bp) of BB101 anneals to the 5' end of the coding sequence of mature IFN- α 2. The 5' segment (39 nucleotides) of BB101 encodes a portion of the STII leader peptide. The ~550 bp PCR product of this reaction was gel purified and used as template for the second PCR reaction. The
 10 second PCR reaction used reverse primer BB100 and forward primer BB11 (5'-CCCCCTCTAGACA TATGAAG AAGAACATCGCATTCCTGCTGGCAT CTATGTTCTGTTTTCTC TATCG-3')
 (SEQ.ID.NO. 7). BB11 adds the remainder of the STII leader peptide and contains an *Nde* I site overlapping the initiator ATG of the STII leader. The ~590 bp product of this reaction was digested with *Nde* I and *Xba* I. The ~100 bp *Nde* I - *Xba* I fragment containing the STII leader sequence and
 15 amino-terminal ~30 bp of IFN- α 2, was gel-purified and ligated with pBBT168 [pUC18::met-IFN- α 2] that had been digested with *Nde* I and *Xba* I, treated with alkaline phosphatase and gel purified. In this step the ~30 bp *Nde* I - *Xba* I amino-terminal segment of the met-IFN- α 2 gene is replaced with the PCR derived ~100 bp *Nde* I - *Xba* I amino-terminal segment of the STII-IFN- α 2 PCR product. The sequence of the resulting pUC18::STII-IFN- α 2 construct was confirmed and that plasmid was
 20 designated pBBT177. For expression in *E. coli*, pBBT177 was digested with *Nde* I and *Eco* RI and the ~570 bp fragment containing the STII-IFN- α 2 gene was gel-purified and cloned into the expression vector pCYB1 under the control of the *tac* promoter. The resulting plasmid was designated pBBT178.

25 B. Expression of rIFN- α 2 in *E. coli*.

pBBT170, which encodes met-IFN- α 2, and parental vector, pCYB1, were transformed into *E. coli* JM109. Experiments with these strains resulted in expression of met-IFN- α 2. Secreted IFN- α 2 is preferable to cytoplasmic met-IFN- α 2 in that secreted IFN- α 2 has the same amino acid sequence as naturally occurring IFN- α 2.

30 For expression of secreted rIFN- α 2, pBBT178 [pCYB1::STII-IFN- α 2] and the parental vector pCYB1 were transformed into *E. coli* W3110. The resulting strains were designated BOB202: W3110(pBBT178) and BOB130: W3110(pCYB1). We performed a series of experiments testing growth and rIFN- α 2 expression of BOB202 in phosphate- or MES- buffered LB media at initial pHs ranging from 5.0 to 7.0. Saturated overnight cultures were diluted to ~0.025 O.D. at A_{600} in buffered
 35 LB containing 100 μ g/ml ampicillin and incubated at 37°C in shake flasks. When culture O.D.s reached ~0.3 - 0.5, IPTG was added to a final concentration of 0.5 mM to induce expression of rIFN- α 2. For initial experiments, cultures were sampled at 0, 1, 3, 5 and ~16 h post-induction. Samples of induced and uninduced cultures were analyzed by SDS-PAGE on precast 14% Tris-glycine polyacrylamide gels stained with Coomassie Blue. In addition to the expected ~19 kDa processed

form of IFN- α 2, we observed a higher than expected molecular weight (~21.5 kDa) form of the protein. The higher molecular weight form could result from lack of proteolytic processing of the STII leader peptide; the molecular weight of the leader peptide is consistent with this hypothesis. Our results indicated that lower pH enhanced accumulation of the correctly sized rIFN- α 2 band. We observed that at or above pH 6.5 the 21.5 kDa form was predominant, while at or below pH 6.0 a band of ~19 kDa, which comigrated with an *E. coli*-expressed rIFN- α 2 standard (Endogen, Inc.), was the predominant form of the protein. At or below pH 6.0, the 19 kDa band accounted for at least 80% and probably greater than 90% of the IFN- α 2 expressed by the *E. coli* cells. Based on these findings, further expression experiments used LB medium buffered with 100 mM MES to a pH of 5.5. Voss et al. (1994) also reported secretion of IFN- α 2 to the *E. coli* periplasm using the STII leader sequence. They also observed that the proportion of rIFN- α 2 present in the 21.5 kDa band was reduced, and the proportion migrating at 19 kDa was increased when culture pH was maintained at 6.7 as compared to 7.0. At pH 7.0, Voss et al. (1994) reported that 10-30% of the STII:IFN- α 2 fusion protein was processed to yield the 19 kDa mature IFN- α 2 protein. The percentage of correctly processed 19 kDa IFN- α 2 protein could be increased to 50-60% by growing the *E. coli* at pH 6.7. However, even at this pH, a substantial amount (40-50%) of the STII: IFN- α 2 fusion protein remained unprocessed, reducing the yield of correctly processed 19 kDa IFN- α 2. Voss et al. (1994) suggested that pH 6.7 was optimal for maximizing the amount of secreted rIFN- α 2 migrating at 19 kDa. Voss et al. (1994) varied several growth parameters to attempt to increase the percentage of correctly processed 19 kDa IFN- α 2 protein to greater than 50-60%, but were unsuccessful. Our data indicate that lowering the pH to below 6.5, and preferably to 5.5 to 6.0, maximizes the ratio of cleaved (19 kDa) to uncleaved (21.5 kDa) rIFN- α 2 product. At these lower pHs, the percent of correctly processed 19 kDa IFN- α 2 is increased to at least 80% and probably 90-100% of the total IFN- α 2 synthesized by the cells.

Cultures expressing rIFN- α 2 were subjected to osmotic shock based on the procedure of Koshland and Botstein (1980). This procedure ruptures the *E. coli* outer membrane and releases the contents of the periplasm into the surrounding medium. Subsequent centrifugation separates the soluble periplasmic components (recovered in the supernatant) from cytoplasmic, insoluble periplasmic, and cell-associated components (recovered in the pellet). Approximately 25 – 50 % of the 19 kDa rIFN- α 2 synthesized by BOB202 was recovered in the supernatant. None of the 21.5 kDa form of rIFN- α 2 was observed in the soluble periplasmic fraction.

C. Large-Scale Expression and Purification of rIFN- α 2 in *E. coli*:

In order to purify the wild type rIFN- α 2 protein, fresh saturated overnight cultures of BOB202 were inoculated at ~0.02 OD @ A_{500} in LB 100mM MES (pH5.5) containing 100 μ g / ml ampicillin. Typically, a 325 ml culture was grown in a 2 liter shake flask at 37°C in a gyrotory shaker water bath at ~160-200 rpm. When cultures reached a density of ~0.3 – 0.4 OD, IPTG was added to a final concentration of 0.5 mM. The induced cultures were then incubated for ~16 h. Cultures were subjected to osmotic shock based on the procedure of Hsuing et. al. (1986). The cells were pelleted by

centrifugation and resuspended at ~25 OD/ml in ice cold 20 % sucrose, 10 mM Tris-HCl (pH 8.0). Resuspended cells were incubated on ice for 15 min and centrifuged at 9500 x g for 10 min. Pellets were then resuspended in ice cold 10 mM Tris-HCl pH (8.0), incubated on ice for 15 min and centrifuged at 9500 x g for 10 min. The resulting supernatant (the osmotic shock lysate) was either
5 processed immediately or stored at -80°C.

rIFN- α 2 was purified as follows. The pH of the supernatant from the osmotic shock was adjusted to 3, centrifuged to remove any precipitate, and loaded onto a 5 ml Pharmacia HiTrap S-Sepharose column equilibrated in 20 mM MES pH 5.0 (Buffer A). The bound proteins were eluted with a linear salt gradient from 0-100% Buffer B (500 mM NaCl, 20 mM MES, 10% ethylene glycol).
10 Column fractions were analyzed by non-reducing SDS-PAGE. rIFN- α 2 eluted at approximately 225-235 mM NaCl. Fractions that were enriched for rIFN- α 2 were pooled and further fractionated on a 1 mL Cu⁺⁺ IMAC (Immobilized Metal Affinity Chromatography) Hi Trap column previously equilibrated in 40 mM sodium phosphate pH 6.0, 1 M NaCl, 0.1% Tween 20. rIFN- α 2 was eluted with a reverse pH gradient from 5.5 to 4.1 in 40 mM sodium phosphate, 1 M NaCl, 0.1% Tween 20.
15 rIFN- α 2 eluted after the gradient reached 100% buffer B, when the pH of the eluate finally reached pH 4.1. Fractions from the Cu⁺⁺ IMAC column that contained purified, properly folded rIFN- α 2 were pooled and stored as frozen aliquots at -80°C. A minor rIFN- α 2 variant was detectable in some of the earlier eluting fractions. This variant, which results from incomplete disulfide formation and is biologically active, has been described previously (Khan and Rai, 1990). Fractions containing this
20 variant were not added to the final pool of purified rIFN- α 2. The final yield of rIFN- α 2, as determined by absorbance at 280 nm and by Bradford analysis, was about 400 μ g from 250 ml of culture. Reduced IFN- α 2 migrates with a slightly larger apparent molecular weight than non-reduced IFN- α 2 when analyzed by SDS-PAGE (Morehead et al., 1984). This apparent molecular weight change is due to the reduction of the native disulfides in IFN- α 2. Our rIFN- α 2 comigrated with the
25 commercial rIFN- α 2 standard under both reducing and non-reducing conditions.

D. *In Vitro* Bioactivity of Wild Type rIFN- α 2.

IFN- α bioactivity can be measured using *in vitro* antiviral assays or cell proliferation inhibition assays. We developed a cell growth inhibition assay to measure bioactivity of wild type rIFN- α 2. The
30 human Daudi B cell line (American Type Culture Collection) is sensitive to the growth inhibiting properties of IFN- α and is routinely used to measure bioactivity of IFN- α (Horoszewicz et al., 1979; Evinger and Pestka, 1981). Daudi cells were maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 μ g/ml streptomycin. For bioassays, the cells were washed three times with RPMI 1640 media and resuspended at a concentration of 4x10⁵ cells/ml in RPMI
35 1640 media containing 10% FBS, 50 units/ml penicillin and 50 μ g/ml streptomycin. Fifty μ l (2x10⁴ cells) of the cell suspension were aliquotted per test well of a flat bottom 96 well tissue culture plate. Serial 3-fold dilutions of the protein samples to be tested were prepared in RPMI 1640 media containing 10% FBS, 50 units/ml penicillin and 50 μ g/ml streptomycin. Fifty μ l of the diluted protein

samples were added to the test wells and the plates incubated at 37°C in a humidified 5% CO₂ tissue culture incubator. Protein samples were assayed in triplicate wells. After 4 days, 20 µl of CellTiter 96 AQueous One Solution Reagent (Promega Corporation) was added to each well and the plates incubated at 37°C in the tissue culture incubator for 1-4 h. Absorbance was read at 490 nm using a microplate reader. Control wells contained media but no cells. Mean absorbance values for the triplicate control wells were subtracted from mean values obtained for each set of triplicate test wells. Serial dilutions of *E. coli*-expressed rIFN-α2 (Endogen, Inc.) were analyzed in parallel. IC₅₀s (the concentration of protein required for half maximal growth inhibition) were calculated for each sample and used to compare bioactivities of the proteins.

Proliferation of the Daudi cell line is strongly inhibited by rIFN-α2, as evidenced by a dose-dependent decrease in absorbance values. Commercial rIFN-α2 (Endogen) and wild type rIFN-α2 prepared by us reached the same maximal level of growth inhibition, within the error of the assay, and had similar mean IC₅₀s of 13-16 pg/ml (Table 4). IC₅₀ values for these proteins ranged from 7-29 pg/ml in assays performed on different days (Table 4); therefore comparisons between proteins were made on samples analyzed on the same day.

EXAMPLE 21

Construction, Expression, Purification and Bioactivity of IFN-α2 Cysteine Muteins

A. Construction of IFN-α2 Cysteine Muteins.

Seventeen mutant IFN-α2 genes were constructed using site-directed PCR-based mutagenesis procedures similar to those described in Example 4. We constructed one mutein in the amino-terminal region proximal to helix A [Q5C]; six muteins in the A-B loop [N45C, Q46C, F47C, Q48C, A50C and 43C44 (an insertion of a cysteine between residues 43 and 44)]; one mutein [D77C] in the short, two residue, BC loop; four muteins in the CD loop [Q101C, T106C, E107C, and T108C]; three muteins in the carboxy-terminal region distal to the E helix [S163C, E165C, and *166C (the addition of a cysteine residue to the natural carboxy-terminus)]. We also constructed muteins C1S and C98S, which eliminate the naturally occurring, but non-essential, C1-C98 disulfide (Lydon et al., 1985; Morehead et al., 1994). The C1S substitution in the amino-terminal region proximal to helix A generates a free cysteine in the C helix (C98), whereas the C98S substitution in the C helix generates a free cysteine in the region proximal to helix A (C1).

For mutagenesis, PCR primer oligonucleotides were designed to incorporate nucleotide changes that resulted in the incorporation of a cysteine residue at the chosen position within the IFN-α2 coding sequence. Where feasible, the mutagenic oligo also was designed to span a nearby restriction site that could be used to clone the mutagenized PCR fragment into an appropriate plasmid. When no useful restriction site was located sufficiently near the position of the mutation, the technique of "mutagenesis by overlap extension" was employed (Horton et al., 1993). The template used for the mutagenic PCR reactions was plasmid pBBT177 (described in Example 20) in which the STII-IFN-α2 gene is cloned as an *Nde* I – *Eco* RI fragment into pUC18. The PCR products were digested with appropriate restriction endonucleases, gel-purified and ligated with pBBT177 vector

DNA that had been cut with those same restriction enzymes, alkaline phosphatase treated, and gel-purified. Transformants from these ligations were grown up and plasmid DNAs isolated and sequenced. The sequence of the entire cloned mutagenized PCR fragment was determined to verify the presence of the mutation of interest, and the absence of any additional mutations that potentially could be introduced by the PCR reaction or by the synthetic oligonucleotide primers.

The substitution mutation Q5C was constructed using the technique of "mutagenesis by overlap extension" as described in Example 4. The initial, or "primary" PCR reactions for the Q5C construction were performed in a 50 μ l reaction volume in 1X Promega PCR buffer containing 1.5 mM $MgCl_2$, each primer at 0.2 μ M, each of dATP, dGTP, dTTP and dCTP at 200 μ M, 1 ng of template plasmid pBBT177 (described in Example 20), 1.5 units of Taq Polymerase (Promega), and 0.25 units of Pfu Polymerase (Stratagene). Reactions were performed in a Robocycler Gradient 96 thermal cycler (Stratagene). The reaction program entailed: 96°C for 3 minutes, 25 cycles of [95°C for 1 minute, 60°C for 1.25 minutes, 72°C for 1 minute] followed by a hold at 6°C. The primer pairs used were [BB125 x BB130] and [BB126 x BB129]. BB125 (5' CTATGC GGCATCAGAGCAGATA >3)(SEQ.ID.NO. 55) anneals to the pUC18 vector sequence ~20 bp upstream of the cloned IFN- α 2 sequence. BB126 (5' TGTGGAATTGTGAGCGGATAAC >3)(SEQ.ID.NO. 56) anneals to the pUC18 vector sequence ~40 bp downstream of the cloned IFN- α 2 sequence. BB129 and BB130 are complementary mutagenic oligonucleotides that change the CAA codon for Q5 to a TGT codon for cysteine. The sequence of BB129 is (5' TGTGATCTGCCTTGTACCCACAGCCTG >3)(SEQ.ID.NO. 57) and the sequence of BB130 is (5' CAGGCTGT GGGTACAAGGCAGATCACA >3)(SEQ.ID.NO. 58). The [BB125 x BB130] and [BB126 x BB129] PCR reactions gave products of the expected sizes: ~140 bp for [BB125 x BB130] and ~560 bp for [BB126 x BB129]. The PCR products were "cleaned up" using the QIAquick PCR Purification Kit (Qiagen) according to the vendor protocol, run out on a 2% agarose gel, gel-purified using a QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol and recovered in 20 μ l 10 mM Tris-HCl (pH 8.5). These two mutagenized fragments were then "spliced" together in the subsequent, or "secondary" PCR reaction. In this reaction 2 μ l of each of the gel-purified PCR products of the primary reactions were used as template and BB125 and BB126 were used as primers. The reaction volume was 100 μ l and 2.5 units of Taq Polymerase and 0.5 units of Pfu Polymerase were employed. Otherwise, the reaction conditions were identical to those used in the primary reactions. An aliquot of the secondary PCR was analyzed by agarose gel electrophoresis and the expected band of ~670 bp was observed. The bulk of the secondary PCR reaction was "cleaned up" using the QIAquick PCR Purification (Qiagen), digested with *Nde* I and *Xba* I (New England BioLabs) according to the vendor protocols, "cleaned up" using the QIAquick PCR Purification Kit and run out on a 2% agarose gel. The ~100 bp *Nde* I - *Xba* I fragment of interest was gel purified using a QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol. This fragment was ligated with pBBT177 (described in Example 20) that had been cut with *Nde* I and *Xba* I, treated with calf intestinal alkaline phosphatase (New England BioLabs) and gel purified. The ligation reaction was used to transform *E. coli* and plasmids from resulting transformants were sequenced to identify a

clone containing the Q5C mutation and having the correct sequence throughout the ~100 bp *Nde* I - *Xba* I segment.

The substitution mutation C1S was constructed and sequence verified using the protocols detailed above for Q5C except that complementary mutagenic primers BB128 (5' AGGCAGATC AGATGCGTAAGC >3)(SEQ.ID.NO. 59) and BB127 (5' GCTTACGCATCTGATCTGCCT >3)(SEQ.ID.NO. 60), which change the TGT codon for C1 to a TCT codon for serine, replaced BB130 and BB129 respectively in the primary PCR reactions. The [BB125 x BB1128] and [BB126 x BB127] PCR reactions gave products of the expected sizes: ~120 bp for [BB125 x BB128] and ~570 bp for [BB126 x BB127].

The substitution mutation N45C was constructed and sequence verified using the protocols detailed above for Q5C with the following differences. Complementary mutagenic primers BB134 (5' CTTTTC GAACTGGCAGCCAACTCCTC >3)(SEQ.ID.NO. 61) and BB133 (5' GAGGAGTTTGGCTGCCAGTTCCAAAAG >3)(SEQ.ID.NO. 62), which change the AAC codon for N45 to a TGC codon for cysteine, replaced BB130 and BB129 respectively in the primary PCR reactions. The [BB125 x BB134] and [BB126 x BB133] PCR reactions gave products of the expected sizes: ~255 bp for [BB125 x BB134] and ~440 bp for [BB126 x BB133]. The product of the secondary PCR reaction was "cleaned up" using the QIAquick PCR Purification (Qiagen), digested with *Bgl* II and *Xba* I (New England BioLabs) according to the vendor protocols, "cleaned up" using the QIAquick PCR Purification Kit and run out on a 2% agarose gel. The ~155 bp *Bgl* II - *Xba* I fragment of interest was gel purified using a QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol. This fragment was ligated with pBBT177 that had been cut with *Bgl* II and *Xba* I, treated with calf intestinal alkaline phosphatase (New England BioLabs) and gel purified. The ligation reaction was used to transform *E. coli* and plasmids from resulting transformants were sequenced to identify a clone containing the N45C mutation and having the correct sequence throughout the ~155 bp *Bgl* II - *Xba* I segment.

The substitution mutation F47C was constructed and sequence verified using the protocols detailed above for N45C with the following differences. Complementary mutagenic primers BB136 (5' TTCAGC CTTTGGCACTGGTTGCCAAA >3)(SEQ.ID.NO. 63) and BB135 (5' TTTGGCAACCAGTGCCAAAAGGCTGAA >3)(SEQ.ID.NO. 64), which change the TTC codon for F47 to a TGC codon for cysteine, replaced BB134 and BB133 respectively in the primary PCR reactions. The [BB125 x BB136] and [BB126 x BB135] PCR reactions gave products of the expected sizes: ~260 bp for [BB125 x BB136] and ~435 bp for [BB126 x BB135].

The insertion mutation 43C44 was constructed and sequence verified using the protocols detailed above for N45C with the following differences. Complementary mutagenic primers BB132 (5' TTCAGCCTT TTGGCACTGGTTGCCAAA >3)(SEQ.ID.NO. 65) and BB131 (5' TTTGGCAACCAGTGCCAAAAGGCTGAA >3)(SEQ.ID.NO. 66), which insert a TGC codon for cysteine between the codons encoding amino acid residues 43 and 44, replaced BB134 and BB133 respectively in the primary PCR reactions. The [BB125 x BB132] and [BB126 x BB131] PCR

reactions gave products of the expected sizes: ~250 bp for [BB125 x BB132] and ~445 bp for [BB126 x BB131].

The substitution mutation Q46C was constructed and sequence verified using the protocols detailed above for N45C with the following differences. Complementary mutagenic primers BB154 (5> AGCCTT TTGGAAACAGTTGCCAACTC >3)(SEQ.ID.NO. 67) and BB153 (5> GAGTTTGGCAACTGTTTCCAAAAGGCT >3)(SEQ.ID.NO. 68), which change the CAG codon for Q46 to a TGT codon for cysteine, replaced BB134 and BB133 respectively in the primary PCR reactions. The primary reactions were performed in a Perkin-Elmer GeneAmp® PCR System 2400 thermal cycler. The reaction program entailed: 95°C for 5 minutes, 30 cycles of [95°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute] followed by 72°C for 7 minutes and a hold at 4°C. The [BB125 x BB154] and [BB126 x BB153] PCR reactions gave products of the expected sizes: ~260 bp for [BB125 x BB154] and ~440 bp for [BB126 x BB153]. The secondary PCR reaction was also performed in a Perkin-Elmer GeneAmp® PCR System 2400 thermal cycler. This reaction program entailed: 96°C for 5 minutes, 25 cycles of [95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute] and a hold at 4°C. Following digestion with *Bgl* II and *Xba* I, the products of this reaction were cleaned up using the QIAquick PCR Purification Kit but not gel purified prior to ligation.

The substitution mutation Q48C was constructed and sequence verified using the protocols detailed above for Q46C with the following differences. Complementary mutagenic primers BB156 (5> GGTTTCA GCCTTACAGAACTGGTTGCC >3)(SEQ.ID.NO. 69) and BB155 (5> GGCAACCAGTTCTGTAAAGGCTGAAACC>3)(SEQ.ID.NO. 70), which change the CAA codon for Q48 to a TGT codon for cysteine, replaced BB154 and BB153 respectively in the primary PCR reactions. The [BB125 x BB156] and [BB126 x BB155] PCR reactions gave products of the expected sizes: ~265 bp for [BB125 x BB156] and ~435 bp for [BB126 x BB155].

The substitution mutation A50C was constructed and sequence verified using the protocols detailed above for Q46C with the following differences. Complementary mutagenic primers BB158 (5> AGGGATGGT TTCACACTTTTGGAACTG >3)(SEQ.ID.NO. 71) and BB157 (5> CAG TTCCAAAAGTGTGAAAC CATCCCT >3)(SEQ.ID.NO. 72), which change the GCT codon for A50 to a TGT codon for cysteine, replaced BB154 and BB153 respectively in the primary PCR reactions. The [BB125 x BB158] and [BB126 x BB157] PCR reactions gave products of the expected sizes: ~270 bp for [BB125 x BB158] and ~440 bp for [BB126 x BB157].

The substitution mutation D77C was constructed and sequence verified using the protocols detailed above for Q5C with the following differences. Complementary mutagenic primers BB138 (5> TAGGAG GGTCTCACACCAAGCAGCAGA >3)(SEQ.ID.NO. 73) and BB137 (5> TCTGCTGCTTGGTGTGAGACCCTCCTA >3)(SEQ.ID.NO. 74), which change the GAT codon for D77 to a TGT codon for cysteine, replaced BB130 and BB129 respectively in the primary PCR reactions. The [BB125 x BB138] and [BB126 x BB137] PCR reactions gave products of the expected sizes: ~350 bp for [BB125 x BB138] and ~345 bp for [BB126 x BB137]. The product of the secondary PCR reaction was "cleaned up" using the QIAquick PCR Purification (Qiagen), digested

with *Bgl* II and *Sal* I (New England BioLabs) according to the vendor protocols, "cleaned up" using the QIAquick PCR Purification Kit and run out on a 2% agarose gel. The ~275 bp *Bgl* II - *Sal* I fragment of interest was gel purified using a QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol. This fragment was ligated with pBBT177 that had been cut with *Bgl* II and *Sal* I, treated with calf intestinal alkaline phosphatase (New England BioLabs) and gel purified. The ligation reaction was used to transform *E. coli* and plasmids from resulting transformants were sequenced to identify a clone containing the D77C mutation and having the correct sequence throughout the ~275 bp *Bgl* II - *Sal* I segment.

The substitution mutation T106C was constructed and sequence verified using the protocols detailed above for D77C with the following differences. Complementary mutagenic primers BB140 (5' > CAGGGGAGTCTCACACACCCCCACCCC >3)(SEQ.ID.NO. 75) and BB139 (5' > GGGGTGGGGTGTGTGAGACTCCCCTG >3)(SEQ.ID.NO. 76), which change the ACA codon for T106 to a TGT codon for cysteine, replaced BB138 and BB137 respectively in the primary PCR reactions. The [BB125 x BB140] and [BB126 x BB139] PCR reactions gave products of the expected sizes: ~435 bp for [BB125 x BB140] and ~260 bp for [BB126 x BB139].

The substitution mutation T108C was constructed and sequence verified using the protocols detailed above for D77C with the following differences. Complementary mutagenic primers BB142 (5' > CTTTCAT CAGGGGACACTCTGTCACCCC >3)(SEQ.ID.NO. 78) and BB141 (5' > GGGGTGACAGAGTGTCCCCTGATGAAG >3)(SEQ.ID.NO. 79), which change the ACT codon for T108 to a TGT codon for cysteine, replaced BB138 and BB137 respectively in the primary PCR reactions. The [BB125 x BB142] and [BB126 x BB141] PCR reactions gave products of the expected sizes: ~440 bp for [BB125 x BB142] and ~250 bp for [BB126 x BB141].

The substitution mutation Q101C was constructed and sequence verified using the protocols detailed above for D77C with the following differences. Complementary mutagenic primers BB162 (5' > CACCCC CACCCACATATCACACAGGC >3)(SEQ.ID.NO. 80) and BB161 (5' > GCCTGTGTGATATGTGGGGTGGGGGTG >3)(SEQ.ID.NO. 81), which change the CAG codon for Q101 to a TGT codon for cysteine, replaced BB138 and BB137 respectively in the primary PCR reactions. The primary reactions were performed in a Perkin-Elmer GeneAmp® PCR System 2400 thermal cycler. The reaction program entailed: 95°C for 5 minutes, 30 cycles of [95°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute] followed by 72°C for 7 minutes and a hold at 4°C. The [BB125 x BB162] and [BB126 x BB161] PCR reactions gave products of the expected sizes: ~ 425 bp for [BB125 x BB162] and ~275 bp for [BB126 x BB161]. The secondary PCR reaction was also performed in a Perkin-Elmer GeneAmp® PCR System 2400 thermal cycler. This reaction program entailed: 96°C for 5 minutes, 25 cycles of [95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute] and a hold at 4°C. Following digestion with *Bgl* II and *Sal* I, the products of this reaction were cleaned up using the QIAquick PCR Purification Kit but not gel purified prior to ligation.

The substitution mutation E107C was constructed and sequence verified using the protocols detailed above for Q101C with the following differences. Complementary mutagenic primers BB164 (5' > CAT CAGGGGAGTACATGTCACCCCCAC >3)(SEQ.ID.NO. 81) and BB163 (5' >

GTGGGGGTGACATGTACTCCCCTG ATG >3)(SEQ.ID.NO. 82), which change the GAG codon for E107 to a TGT codon for cysteine, replaced BB162 and BB161 respectively in the primary PCR reactions. The [BB125 x BB164] and [BB126 x BB163] PCR reactions gave products of the expected sizes: ~ 440 bp for [BB125 x BB164] and ~255 bp for [BB126 x BB163].

5 The substitution mutation C98S was constructed and sequence verified using the protocols detailed above for Q101C with the following differences. Complementary mutagenic primers BB160 (5> CCC CTGTATCACAGAGGCTTCCAGGTC >3)(SEQ.ID.NO. 83) and BB159 (5> GACCTGGAAGCCTCTGTGATACA GGGG >3)(SEQ.ID.NO. 84), which change the TGT codon for C98S to a TCT codon for serine, replaced BB162 and BB161 respectively in the primary PCR reactions. The [BB125 x BB160] and [BB126 x BB159] PCR reactions gave products of the expected sizes: ~ 415 bp for [BB125 x BB160] and ~ 285 bp for [BB126 x BB159].

 The cysteine substitution mutation S163C was constructed as follows. The mutagenic reverse oligonucleotide BB143 (5> CGCGAATTCTTATTCCTTACATCTTAACTTTC >3)(SEQ.ID.NO. 85) was designed to change the codon AGT for serine at position 163 to a TGT encoding cysteine and to span the nearby *Eco* RI site. This oligo was used in PCR with the forward, non-mutagenic, primer BB125. A 50 µl PCR reaction was performed in 1X Promega PCR buffer containing 1.5 mM MgCl₂, each primer at 0.2µM, each of dATP, dGTP, dTTP and dCTP at 200 µM, 1 ng of template plasmid pBBT131 1.5 units of Taq Polymerase (Promega), and 0.25 units of Pfu Polymerase (Stratagene). Reactions were performed in a Robocycler Gradient 96 thermal cycler (Stratagene). The reaction program entailed: 96°C for 3 minutes, 25 cycles of [95°C for 1 minute, 60°C for 1.25 minutes, 72°C for 1 minute] followed by a hold at 6°C. A 5 µl aliquot of the PCR reaction was analyzed by agarose gel electrophoresis and found to produce a single fragment of the expected size ~ 610 bp. The remainder of the reaction was "cleaned up" using the QIAquick PCR Purification (Qiagen) according to the vendor protocol, digested with *Sal* I and *Eco* RI (New England BioLabs) according to the vendor protocols, ethanol-precipitated, resuspended in 20 µl of 10 mM Tris-HCl pH 8.5 and run out on a 2% agarose gel. The ~42 bp *Sal* I - *Eco* RI fragment of interest was gel purified using a QIAEX II Gel Extraction Kit (Qiagen) according to the vendor protocol. This fragment was ligated with pBBT132 that had been cut with *Sal* I and *Eco* RI, treated with calf intestinal alkaline phosphatase (New England BioLabs) and gel purified. The ligation reaction was used to transform *E. coli* and plasmids from resulting transformants were sequenced to identify a clone containing the E107C mutation and to have the correct sequence throughout the ~42 bp *Sal* I - *Eco* RI segment.

 A mutation was also constructed that added a cysteine following the carboxyterminal amino acid of the IFN-α2 coding sequence. This mutant, termed *167C was constructed using the protocols described above for the construction of the S163C mutant with the following differences. The mutagenic reverse oligonucleotide BB144 (5> CGCGAATTCTTAACATTCCTTACTTCTTAACTTTC >3)(SEQ.ID.NO. 86) which adds a TGT codon for cysteine between the GAA codon for E165 and the TAA stop codon and spans the nearby *Eco* RI site was used in the PCR reaction in place of BB143.

 The substitution mutation E165C was constructed and sequence verified using the protocols detailed above for S163C with the following differences. The mutagenic reverse oligonucleotide BB165 (5>

CGCGAATTCTTA ACACTTACTTCTTAAACT >3)(SEQ.ID.NO. 87) which changes the GAA codon for E165 to a TGT codon for cysteine and spans the nearby *Eco* RI site was used in the PCR reaction in place of BB143. The PCR reaction was performed in a Perkin-Elmer GeneAmp® PCR System 2400 thermal cycler. The reaction program entailed: 95°C for 5 minutes, 30 cycles of [95°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute] followed by 72°C for 7 minutes and a hold at 4°C. Following digestion with *Eco* RI and *Sal* I, the products of this reaction were cleaned up using the QIAquick PCR Purification

For expression in *E. coli* as proteins secreted to the periplasmic space, the STII-IFN- α 2 genes encoding the 17 muteins were excised from the pUC18-based pBBT177 derivatives as *Nde* I – *Eco* RI fragments of ~590 bp and subcloned into the pCYB1 expression vector that had been used to express wild type STII-IFN α -2. For expression experiments, these plasmids were introduced into *E. coli* W3110.

Using procedures similar to those described here, one can construct other cysteine muteins of IFN- α 2. The cysteine muteins can be substitution mutations that substitute cysteine for a natural amino residue in the IFN- α 2 coding sequence, insertion mutations that insert a cysteine residue between two naturally occurring amino acids in the IFN- α 2 coding sequence, or addition mutations that add a cysteine residue preceding the first amino acid, C1, of the IFN- α 2 coding sequence or add a cysteine residue following the terminal amino acid residue, E165, of the IFN- α 2 coding sequence. The cysteine residues can be substituted for any amino acid, or inserted between any two amino acids, anywhere in the IFN- α 2 coding sequence. Preferred sites for substituting or inserting cysteine residues in IFN- α 2 are in the region preceding Helix A, the A-B loop, the B-C loop, the C-D loop, the D-E loop and the region distal to Helix E. Other preferred sites are the first or last three amino acids of the A, B, C, D and E Helices. Preferred residues in these regions for creating cysteine substitutions are D2, L3, P4, T6, H7, S8, Q20, R22, K23, S25, F27, S28, K31, D32, R33, D35, G37, F38, Q40, E41, E42, F43, G44, K49, T52, N65, S68, T69, K70, D71, S72, S73, A74, A75, D77, E78, T79, Y89, Q90, Q91, N93, D94, E96, A97, G102, V103, G104, V105, P109, M111, K112, E113, D114, S115, K131, E132, K133, K134, Y135, S136, A139, S152, S154, T155, N156, L157, Q158, E159, S160, L161, R162, and K164. Cysteine residues also can be inserted immediately preceding or following these amino acids. Another preferred site for adding a cysteine residue would be preceding C1, which we refer to as *-1C.

One also can construct IFN- α 2 muteins containing a free cysteine by substituting another amino acid for one of the naturally-occurring cysteine residues in IFN- α 2. The naturally-occurring cysteine residue that normally forms a disulfide bond with the substituted cysteine residue is now free. The non-essential cysteine residue can be replaced with any of the other 19 amino acids, but preferably with a serine or alanine residue. A free cysteine residue also can be introduced into IFN- α 2 by chemical modification of a naturally occurring amino acid using procedures such as those described by Sytkowski et al. (1998).

Using procedures similar to those described in Examples 20 – 22, one can express the proteins in *E. coli*, purify the proteins, PEGylate the proteins and measure their bioactivities in an *in vitro* bioassay. The IFN- α 2 muteins also can be expressed in eukaryotic cells such as insect or

mammalian cells, using procedures similar to those described in Examples 16-20, or related procedures well known to those skilled in the art.

B. *E. coli* Expression of rIFN- α 2 Cysteine Muteins.

5 To assess expression, cultures of the rIFN- α 2 muteins were grown and induced as described above for wild type rIFN- α 2. Typically, 45 ml cultures were grown in 250 ml shake flasks at 37°C in a gyrotory shaker water bath at ~180-200 rpm. We observed that vigorous aeration of shake flask cultures results in reduced levels of IFN- α 2 protein in the supernatants of the osmotic shock lysates. Therefore we routinely used conditions that were sub-optimal for aeration but preferable for soluble
10 rIFN- α 2 and rIFN- α 2 mutein production. The induced cultures were incubated for ~16 h, harvested and subjected to osmotic shock as detailed above for wild-type rIFN- α 2 with the exception that cystine was added to a final concentration of 5 mM to the buffers used for the osmotic shock procedure. Adding cystine to the osmotic shock buffers resulted in significantly improved chromatographic properties for the first two muteins analyzed, Q5C and S163C. Interferon muteins not treated with
15 cystine consistently eluted from the S-Sepharose column as broad bands, which, when analyzed by non-reducing SDS-PAGE, showed multiple molecular weight species at and around the expected monomer molecular weight. These species most likely represent misfolded or incompletely folded interferon variants. In contrast, interferon muteins treated with cystine during the osmotic shock procedure eluted from the S-Sepharose column as sharp bands, which, when analyzed by non-
20 reducing SDS-PAGE, consisted of only one interferon species that co-migrated with the interferon wild type standard. Recoveries of the purified interferon muteins from the S-Sepharose column also were 1.5- to 2-fold greater when cystine was included in the osmotic shock buffers. Based upon these results, 5 mM cystine was added to the osmotic shock buffers used to purify all the cysteine muteins.

SDS-PAGE analysis of the osmotic shock supernatants of the muteins showed most to have
25 reduced (as compared to wild type) levels of the 19 kDa rIFN- α 2 band. Two muteins, Q5C and S163C, were expressed at levels equivalent to wild type interferon, and several hundred micrograms of each of these muteins were readily purified as detailed below. Eight muteins (C1S, 43C44, N45C, Q46C, Q48C, A50C, D77C and T108C) were essentially undetectable in osmotic shock supernatants. The remaining seven muteins (F47C, C98S, Q101C, T106C, E107C, E165C, *166C) were detected at
30 varying levels (as compared to wild type) in osmotic shock supernatants. Some of these muteins (T106C, C98S, E107C, and Q101C) were purified from osmotic shock supernatants, but only small quantities of the pure proteins were recovered. Certain muteins, C98S, Q101C, T106C, E107C and *166C, were expressed at relatively high levels but accumulated primarily in an insoluble form, presumably in the periplasm. These proteins comigrated with wild type rIFN- α 2 standards under
35 reducing conditions indicating that the STII leader had been removed. Qualitative assessments of relative expression levels of the muteins are summarized in Table 4.

C. Purification of rIFN- α 2 cysteine muteins.

In order to purify the rIFN- α 2 muteins, typically, a 325 ml culture in a 2 liter shake flask, or a 500 ml culture in a 2 liter baffled shake flask, were grown at 37°C in a gyrotory shaker water bath (New Brunswick Scientific) ~170-220 rpm. Cultures were grown, induced, harvested, and subjected to osmotic shock as described in Example 20. Resulting osmotic shock supernatants were processed immediately or stored at -80°C.

The soluble IFN- α 2 muteins in the osmotic shock supernatants were purified using S-Sepharose and Cu⁺⁺ IMAC chromatography as detailed above for purification of wild type rIFN- α 2. All of the muteins tested bound tightly to the copper column and eluted under conditions similar to wild-type rIFN- α 2. This result suggests that the conformations of the cysteine muteins are similar to that of native rIFN- α 2, at least in the regions that comprise the metal-binding pocket.

Non-reducing SDS-PAGE analysis of the purified Q5C, C98S, Q101C, T106C, E107C, S163C, and *166C cysteine muteins showed that the muteins were recovered predominantly as monomers, migrating at the expected molecular weight of ~ 19 kDa. C98S migrated with a slightly higher molecular weight than the other rINF- α 2 muteins due to the absence of the native Cys1-Cys-98 disulfide bond. Some of the purified muteins contained small amounts of disulfide-linked rIFN- α 2 dimers. The molecular weights of the dimer species were approximately 37-38 kDa.

D. Bioactivities of rIFN- α 2 Cysteine Muteins.

Biological activities of the purified Q5C and S163C rIFN- α 2 cysteine muteins were measured in the Daudi growth inhibition assay described in Example 20. Protein concentrations were determined using Bradford or BCA protein assay kits (Bio-Rad Laboratories and Pierce). Commercial wild type rIFN- α 2 and rIFN- α 2 prepared by us were analyzed in parallel on the same days to control for interday variability in the assays. The muteins inhibited proliferation of Daudi cells to the same extent as the wild type rIFN- α 2 control proteins, within the error of the assay. The mean IC₅₀ for the Q5C mutein was 13 pg/ml, which is similar to the mean IC₅₀ s of the wild type rIFN- α proteins. The mean IC₅₀ for the S163C protein was 27 pg/ml. These data are summarized in Table 4.

Table 4.
Expression and *in vitro* Bioactivities of IFN- α 2 Cysteine Muteins

IFN- α 2 Protein	Mutation Location	Relative Expression		Mean IC ₅₀ (pg/ml)	IC ₅₀ Range ¹ (pg/ml)
		Total Cellular ¹	Percent Soluble ²		
rIFN- α 2 ⁴	-	-	-	16 +/- 7	8-29 (n=10)
rIFN- α 2 ⁵	-	++++	~ 33	13 +/- 4	7-19 (n=10)
C1S	N-terminal region ⁶	+/-	0		
Q5C	N-terminal region	++++	~ 20	13	9,11,15,18
43C44	A-B loop	++	0		
N45C	A-B loop	++	0		
Q46C	A-B loop	+/-	0		
F47C	A-B loop	++++	~5		
Q48C	A-B loop	+/-	0		
A50C	A-B loop	+/-	0		
D77C	B-C loop	+/-	0		

IFN- α 2 Protein	Mutation Location	Relative Expression		Mean IC ₅₀ (pg/ml)	IC ₅₀ Range ³ (pg/ml)
		Total Cellular ¹	Percent Soluble ²		
C98S	C-helix ⁷	+++++	~ 5-10		
Q101C	C-D loop	+++++	~ 5-10		
T106C	C-D loop	+++++	~ 5-10		
E107C	C-D loop	+++++	~ 5-10		
T108C	C-D loop	+/-	0		
S163C	C-terminal region	++++	~ 33	27 +/- 8	18-40 (n=6)
E165C	C-terminal region	+++	~ 20		
*166C	C-terminus	+++	~ 20		

¹ Relative accumulation of the IFN- α 2 protein in whole cell extracts

² Portion of the IFN- α 2 protein in the osmotic shock supernatant, estimated from SDS-PAGE gels

³ IC₅₀ values from individual experiments

5 ⁴ Commercial wild type rIFN- α 2 (Endogen, Inc.)

⁵ Wild type rIFN- α 2 prepared by Bolder Biotechnology, Inc.

⁶ Mutation creates a free cysteine (C98) in the C-helix

⁷ Mutation creates a free cysteine (C1) in the N-terminal region

10

EXAMPLE 22

PEGylation, Purification and Bioactivity of PEG-Q5C and PEG-S163C

A. PEGylation of IFN- α Cysteine muteins.

A small-scale PEGylation experiment was performed with the purified rIFN- α 2 cysteine
 15 muteins to identify conditions that allowed the proteins to be monoPEGylated at the free cysteine
 residue. Over-reduction of the proteins was monitored by non-reducing SDS-PAGE, looking for a
 shift to a higher than expected apparent molecular weight as a result of protein unfolding, or for the
 appearance of multiple PEGylated species generated as the result of native disulfide reduction. Initial
 titration experiments were performed with the Q5C protein. One μ g aliquots of purified Q5C were
 20 incubated with increasing concentrations of TCEP [Tris (2-carboxyethyl) phosphine]-HCl at room
 temperature in 100 mM Tris, pH 8.5 in the presence of varying amounts of excess 5 kDa maleimide-
 PEG. After 60 min, the reactions were stopped and immediately analyzed by non-reducing SDS-
 PAGE. The amounts of TCEP and PEG reagent that yielded significant amounts of monoPEGylated
 Q5C protein (molecular weight of approximately 28 kDa by non-reducing SDS-PAGE), without
 25 modifying wild type rIFN- α 2, were used for further experiments. The titration experiments indicated
 that a 10-fold molar excess of TCEP and 20-fold excess of 5 kDa maleimide PEG gave around 60%
 monoPEGylated protein without detectable di or tri-PEGylated protein, or modification of wild type
 rIFN- α 2.

These conditions also were used to PEGylate several other rIFN- α 2 muteins. One μ g
 30 aliquots of purified wild type and the rIFN- α 2 muteins (Q5C, T106C, E107C, S163C) were incubated
 for 1 hour with a 10-fold molar excess TCEP and a 20-fold molar excess of 5 kDa maleimide PEG at
 pH 8.5 at room temperature. The four muteins were monoPEGylated to varying degrees (estimated to
 be from 30-60%) based on SDS-PAGE analysis of the reaction mixtures. Wild-type rIFN- α 2 showed
 no detectable PEGylation under these conditions. Control experiments indicated that the Q5C,

T106C, E107C and S163C cysteine muteins needed to be reduced with TCEP to be PEGylated. These data indicate that the PEG molecule is attached to the cysteine residue introduced into the Q5C, T106C, E107C and S163C proteins.

5 **B. Preparation and Purification of PEG-Q5C IFN- α 2 and PEG-S163C:**

Larger quantities of the Q5C and S163C muteins were PEGylated so that biological activities of the PEGylated proteins could be measured. For the Q5C protein, the PEGylation conditions used for the small-scale experiments were scaled to 140 μ g protein to give sufficient material for purification and characterization. The larger PEGylation reaction was performed for 1 hr at room
10 temperature, diluted 10X with 20 mM MES, pH 5.0, adjusted to pH 3.0, and then loaded quickly onto an S-Sepharose column using conditions similar to those described for initial purification of the rIFN- α 2 muteins. The presence of the PEG moiety decreased the protein's affinity for the resin, allowing the PEGylated protein to be separated from the non-PEGylated protein. The chromatogram from the S-Sepharose column showed two major protein peaks eluting at approximately 190 mM NaCl and 230
15 mM NaCl. The early eluting major peak (eluting at approximately 190 mM NaCl) was determined to be mono-PEGylated Q5C by SDS-PAGE. The apparent molecular weight of monoPEGylated Q5C is approximately 28 kDa by SDS-PAGE. The later eluting major peak (eluting at approximately 230 mM NaCl) was determined to be unreacted Q5C protein. Fractions from the early eluting peak containing predominantly PEG-Q5C, were pooled and used for bioactivity measurements.

20 The S163C cysteine mutant was PEGylated at a 90 μ g scale and purified using protocols essentially identical to those described for PEG-Q5C.

C. Bioactivities of PEG-Q5C and PEG-S163C Cysteine Muteins:

Biological activity of the purified PEG-Q5C protein was measured in the Daudi cell assay
25 described in Example 20. Concentration of the protein was determined using a Bradford dye binding assay. The PEG-Q5C protein showed a similar dose-response curve and reached the same level of maximal growth inhibition as wild type rIFN- α 2 and the non-modified Q5C protein, within the error of the assay. The mean IC₅₀ for the PEG-Q5C mutein was ~ 22 pg/ml, which is within 2-fold of the IC₅₀ values determined for wild type IFN- α 2 and the unmodified Q5C proteins analyzed on the same
30 days (Table 5). Bioactivity of the PEG-Q5C protein is significantly greater than that of rIFN- α 2 that has been PEGylated with non-specific, amine-reactive PEG reagents. The latter protein has an IC₅₀ of 164 pg/ml in the Daudi cell assay (Monkarsh et al., 1997). These data are summarized in Table 5.

Bioactivity experiments also were performed with the PEG-S163C protein. The PEG-S163C protein also was biologically active and inhibited Daudi cell proliferation to the same extent as wild
35 type rIFN- α 2, within the error of the assay. The average IC₅₀ for the PEG-S163C protein was about 42 pg/ml, which is better than the amine-Pegylated IFN- α

Table 5. Bioactivity of PEG-Q5C IFN- α

IFN- α Protein	Mean EC ₅₀ (pg/ml)	EC ₅₀ Range ¹ (pg/ml)		
		Exp A	Exp B	Exp C
Endogen rIFN- α	13	9	11	20
rIFN- α ²	12	10	10	16
Q5C	12	8.5	11	18
PEG-Q5C	22	18	18	30
Amine-PEGylated-IFN- α ³	164	-	-	-

¹ Data from three experiments.² rIFN- α 2 prepared by Bolder Biotechnology, Inc.³ Data from Monkarsh et al. (1997)

5

Example 23

In vivo efficacy of the PEGylated GH cysteine muteins can be tested in hypophysectomized (HYPOX) rats. This is a well-characterized model of GH deficiency (Cox et al., 1994; Clark et al., 1996). GH stimulates body weight gain and bone and cartilage growth in HYPOX rats (Cox et al., 1994; Clark et al., 1996). Hypophysectomized Sprague-Dawley rats can be purchased from a commercial supplier such as Charles River (Wilmington, MA). Typically, rats are hypophysectomized between 40 and 50 days of age and weigh approximately 120g. Groups of 8 rats should receive subcutaneous injections of rhGH, PEG-Cys-GH or placebo (vehicle solution) at specified intervals and weight gain measured daily over a 10 day period. Rats should be weighed daily at the same time per day to eliminate possible variables associated with feeding. In addition to overall weight gain, bone growth (tibial epiphysis width) can be measured. At time of sacrifice, the right and left proximal tibial epiphyses can be removed and fixed in formalin. The fixed tibias can be split at the proximal end in a sagittal plane, stained with silver nitrate and exposed to a strong light (Greenspan et al., 1949). The width of the cartilaginous epiphysal plate can be measured using a stereomicroscope equipped with a micrometer eyepiece. Ten measurements should be made for each epiphysis and the means +/- SEM for the combined values for the left and right tibias should be calculated. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. P<0.05 should be considered significant.

Efficacy of the GH cysteine muteins modified with 10 kDa or 20 kDa PEGs can be tested by administering the proteins to the rats daily, every other day, every third day, every fourth day or following a single injection. Five μ g of non-PEGylated hGH administered twice a day (10 μ g BID) by subcutaneous injection gives a strong growth response in the HYPOX rat model (Cox et al., 1994; Clark et al., 1996). In initial experiments different groups of rats should receive subcutaneous injections of 0.08, 0.4, 2, 10, or 50 μ g of the PEGylated Cys-GH proteins/injection/rat. Control rats should receive vehicle solution only. Additional control groups should receive non-PEGylated rhGH (10 μ g/BID) and 10 μ g non-PEGylated hGH using the same dosing regimen as the PEGylated Cys-GH proteins. Administration of the PEGylated GH cysteine muteins to the HYPOX rats should result in an increase in body weight gain and tibial epiphysis width growth compared to the vehicle-treated group.

Efficacy of the PEGylated GH cysteine muteins also can be tested in rodent models of cachexia. Dexamethasone (DEX) can be administered to the rats to induce weight loss. Groups of normal Sprague-Dawley rats (200 – 225g) should receive daily subcutaneous injections of dexamethasone (200 µg/rat; approximately 1 mg/kg). This amount of dexamethasone should induce a loss of approximately 5-6g over an 8d period. Vehicle or varying doses of the PEGylated GH cysteine muteins can be administered to the rats once, daily, every other day, every third day or every fourth day in different experiments. Different groups of rats should receive subcutaneous injections of 0.08, 0.4, 2, 10, or 50 µg of the PEGylated Cys-GH proteins/injection/rat. Additional controls should include a group of rats that will receive no DEX or injections, a group of rats that receives DEX and non-PEGylated rhGH (10 µg BID) and a group of rats that receives DEX and non-PEGylated rhGH (10 µg daily, every other day, every third day, or every fourth day, depending upon the experiment, i.e., frequency that the PEGylated GH cysteine mutein is administered). Animals should be weighed daily. Food and water consumption should be monitored daily. At time of sacrifice, internal organs should be weighed. Statistical analyses should be performed as described for the HYPOX rat studies. Animals treated with the PEGylated GH cysteine muteins should lose less weight than the vehicle-treated animals.

In vivo efficacy of the PEGylated EPO cysteine muteins can be measured in normal rats by demonstrating that the proteins stimulate increases in hemocrit and erythropoiesis compared to vehicle-treated animals. EPO stimulates a significant increase in hematocrit in rats when dosed on a daily basis (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998). Sprague-Dawley rats can be purchased from a commercial supplier such as Charles River (Wilmington, MA). Groups of 5 rats should receive subcutaneous injections of BV rEPO, PEGylated EPO cysteine mutein or placebo (vehicle solution) at specified intervals for up to five days. On day 6 the animals should be sacrificed and blood samples collected for hematocrit and complete blood cell count (CBC) analysis, which can be performed by a commercial laboratory. Hematopoietic tissues (liver and spleen) should be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased erythropoiesis. Bone marrow should be removed from various long bones and the sternum for unit particle preparations and histopathologic analysis to look for evidence of increased erythropoiesis. Comparisons between groups should be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. $P < 0.05$ should be considered significant. The PEGylated EPO cysteine muteins should stimulate increases in hematocrit and erythropoiesis in the rats compared to the vehicle-injected animals. Efficacy of the PEGylated EPO cysteine muteins modified with 10 kDa or 20 kDa PEGs can be tested when administered once, every other day or every third day. 100 IU/kg (~ 800 ng/kg) of non-PEGylated EPO administered once per day (160 ng SID/200g rat) by subcutaneous injection gives a significant increase in hematocrit (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998). In initial experiments different groups of rats should receive subcutaneous injections of 0.32, 1.6, 8, 40 or 160 ng of the PEGylated EPO cysteine muteins. Control rats should receive vehicle solution only. Additional control groups should receive non-

PEGylated rEPO (160 ng/SID) and 160 ng non-PEGylated rEPO using the same dosing regimen as the PEGylated EPO cysteine muteins.

Efficacy of the PEGylated EPO muteins also can be tested in chemotherapy-induced anemia models. Cisplatin-induced anemia is a well-characterized rodent model of chemotherapy-induced anemia and has direct relevance to the human clinical setting. rEPO reverses the anemia in this model when administered at daily doses of 100 Units/kg (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al. 1998). Sprague-Dawley rats (~200g) should be treated on day 0 with an intraperitoneal injection of Cisplatin (3.5mg/kg) to induce anemia and randomized to various treatment groups. Efficacy of the PEGylated EPO cysteine muteins modified with 10 kDa or 20 kDa PEGs can be tested when administered once (on day 1), every other day or every third day. Different groups of rats should receive subcutaneous injections of 0.32, 1.6, 8, 40 or 160 ng/injection of the PEGylated EPO cysteine muteins. Rats should be injected with the test compounds for up to 8 days. One control group of rats should receive daily subcutaneous injections of rEPO (100 Units/kg). Another control group should not receive the initial Cisplatin injection but should receive injections of saline using the same dosing schedules used for the PEGylated EPO cysteine muteins. On day 9 the rats should be sacrificed and blood and tissue samples obtained for comprehensive CBC and histopathology analyses. The PEGylated EPO cysteine muteins should stimulate increases in hemocrit and erythropoiesis in the rats compared to the vehicle-injected control group.

IFN- α biological activity is relatively species-specific, which limits the range of preclinical animal models that can be studied. One model that can be used to measure *in vivo* efficacy of PEGylated IFN- α 2 cysteine muteins is inhibition of human tumor xenograft growth in athymic nude mice. Human IFN- α 2 is not active on mouse cells and inhibition of human tumor xenograft growth in nude mice occurs through a direct antiproliferative effect on the human tumor cells. IFN- α 2 inhibits growth of a variety of primary human tumor xenografts and human tumor cell lines in athymic mice (Balkwill et al., 1985; Balkwill, 1986; Johns et al., 1992; Lindner and Borden, 1997). The primary endpoint for the studies should be tumor volume in treated mice. We expect to find that the administration of the PEGylated IFN- α 2 cysteine muteins inhibits tumor growth (as measured by tumor volume) in the mice relative to vehicle-treated animals. Athymic nude mice can be purchased from a commercial vendor such as Charles River. Each mouse should be injected with 2×10^6 NIH-OVCAR-3 or MCF-7 tumor cells (the cell lines are available from the American Type Culture collection) on day 0 and randomly assigned to test groups, consisting of ten mice each. The tumor cells should be injected into the dermis overlying the mammary gland nearest the axillae. The different test groups should receive subcutaneous injections of varying doses of wild type rIFN- α 2, 10 kDa-PEGylated IFN- α 2 cysteine mutein, 20 kDa-PEGylated IFN- α 2 cysteine mutein or placebo (vehicle solution) at specified intervals: every day (SID), every other day (EOD) or every third day (ETD). Tumor volumes should be determined at 4 day intervals by measuring the length and width of the tumors with calipers, as described by Linder and Borden (1997). At time of sacrifice, the tumors should be excised and weighed. Mean tumor volumes \pm SEM for each test group should be calculated for each sampling point. Comparisons between groups should be made using a Students T

test for single comparisons and one-way analysis of variance for multiple comparisons. Five μg of non-PEGylated IFN- $\alpha 2$ administered once per day by subcutaneous injection inhibits growth of NIH-OVCAR-3 cells and MCF-7 cells in athymic mice by 80% after 6 weeks (Lindner and Borden, 1997). Either cell line can be used for these studies. The NIH-OVCAR-3 line (available from the ATCC) does not require estrogen for growth, as do the MCF-7 cells. Xenograft experiments with MCF-7 cells require that the mice be oophorectomized and implanted with estrogen pellets (Lindner and Borden, 1997). In initial experiments, different groups of mice should receive subcutaneous injections of 1 or 5 μg per injection of rIFN- $\alpha 2$, 10-kDa-PEGylated IFN- $\alpha 2$ cysteine mutein or 20-kDa-PEGylated IFN- $\alpha 2$ cysteine mutein using every day, every other day or every third day dosing schedules. Dosing should begin on day 2 following injection of the tumor cells into the mice. Control mice should receive vehicle solution only. In the every other day and every third day dosing experiments, an additional positive control group should receive daily subcutaneous injections of 5 μg unmodified rIFN- $\alpha 2$.

15

References

- Abdel-Meguid, S.S., Shieh, H-S., Smith W.W., Dayringer, H.E., Violand, B.N. and Bcntle, L.A. (1987) *Proc. Natl. Acad. Sci. USA* 84: 6434-6437.
- 5 Abrahmsen, L., Moks, T., Nilsson, B. and Uhlen, M. (1986) *Nucleic Acids Res.* 14:7487-7500.
- Abuchowski, A., Kazo, G.M., Verhoest, C.R., van Es, T., Kafkewitz, D., Nucci, M.L., Viau, A.T. and Davis, F.F. (1984) *Cancer Biochem. Biophys.* 7: 175-186.
- Alt, F.W., Kellems, R.E., Bertino, J.R., and Schimke, R.T. (1978) *J. Biol. Chem.* 253:1357-1370.
- 10 Ayres, M.D., Howard, S.C., Kuzio, J., Iopez-Ferber, M., and Possee, R.D. (1994) *Virology* 202:586-605.
- Barik, S. (1993) in "Methods in Molecular Biology", White, B.A., ed. (Humana Press, Totawa, NJ), 15: 277-286.
- 15 Bazan, F. (1991) *Immunology Today* 11: 350-354.
- Bazan, J.F. (1992) *Science* 257: 410-411.
- Becker, G.W. and Hsiung, H.M. (1986) *FEBS Lett.* 204: 145-150.
- Bewley et al., (1969) *Biochem.* 8: 4701-4708.
- Bill, R.M., Winter, P.C., McHale, C.M., Hodges, V.M., Elder, G.E., Caley, J., Flitsch, S.L.,
- 20 Bicknell, R. and Lappin, T.R.J. (1995) *Biochem. Biophys. Acta* 126: 35-43.
- Blatt, L.M., Davis, J.M., Klein, S.B. and Taylor, M.W. (1996) *J. Interferon and Cytokine Research* 16: 489-499.
- Boissel, J.-P., Lee, W.-R., Presnell, S.R., Cohen, F.E. and Bunn, H.F. (1993) *J. Biol. Chem.* 268:15983-15993.
- 25 Bollag, D.M., Rozycki, M.D. and Edelstein, S.J. (1996) *Protein Methods*, 415 pages, Wiley-Liss, NY, NY.
- Braxton, S.M. (1998) U.S. Patent 5,766,897.
- Chamow & Ashkenazi (1996), *Trends in biotech* 14:52-60.
- Chang, C.N., Rey, B., Bochner, B., Heyneker, H. and Gray, G. (1987) *Gene*: 189-196.
- 30 Cheah, K-C., Harrison, S., King, R., Crocker, L., Wells, J.R.E. and Robins, A. (1994) *Gene*, 138: 9-15.
- Clark, R., Olson, K., Fuh, G., Marian, M., Mortensen, D., Teshima, G., Chang, S., Chu, H., Mukku, V., Canova-Davis, E., Somers, T., Cronin, M., Winkler, M. and Wells, J. A. (1996) *J. Biol. Chem.* 271: 21969-21977.
- 35 Cox, G.N. and McDermott, M.J. (1994) WO 9412219.
- Cox, G.N., McDermott, M.J., Merkel, E., Stroh, C.A., Ko, S.C., Squires, C.H., Gleason, T.M. and Russell, D. (1994) *Endocrinology* 135: 1913-1920.
- Cox, G.N. and Russell, D. (1994) WO 9,422,466.
- Crouse, G.F., McEwan, R.N., and Pearson, M.L. (1983) *Mol. Cell. Biol.* 3:257-266.
- 40 Cunningham, B.C. and Wells, J.A. (1989) *Science* 244: 1081-1085.

- Cunningham, B.C., Jhurani, P., Ng, P. and Wells, J.A. (1989) *Science* 243: 1330-1336.
- Cunningham, B.C., Ultsch, M., de Vos, A.M., Mulkerrin, M.G., Clauser, K.R. and Wells, J.A. (1991) *Science* 254: 821-825.
- Daopin, S., Piez, K.A., Ogawa, Y., and Davies, D.R. (1992) *Science* 257: 369-373.
- 5 Davis, S., Aldrich, T.H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N.Y. and Yancopoulos, G.D. (1993) *Science* 260: 1805-1808.
- Davies, A.H. (1995) *Curr. Opin. Biotechnol.* 6:543-547.
- DeChiara, T.M., Erlitz, F. and Tarnowski, S.J. (1986) *Methods Enzymology*, 119: 403-15.
- 10 de la Llosa, P., Chene, N. and Martal, J. (1985) *FEBS Letts.* 191: 211-215.
- Deneffe, P., Kovarik, S., Ciora, T., Gosselet, M., Benichou, J.-C., Latta, M., Guinet, F., Ryter, A. and Mayaux, J.-F. (1989) *Gene* 85, 499-510.
- de Vos, A.M., Ultsch, M. and Kossiakoff, A.A. (1992) *Science* 255: 306-312.
- Diederichs, K., Boone, T. and Karplus, A. (1991) *Science* 154: 1779-1782.
- 15 Evinger, M. and Pestka, S. (1981) *Methods Enzymol.* 79:362-368.
- Fuh, G., Cunningham, B.C., Fukunaga, R., Nagata, S., Goeddel, D.V. and Wells, J.A. (1992) *Science* 256: 1677-1680.
- Fujimoto, K., Fukuda, T., and Marumoto, R. (1988) *J. Biotechnol.* 8:77-86.
- Geisse, S., Gram, H., Kleuser, B. and Kocher, H.P. (1996) *Prot. Express. Purif.* 8:271- 282.
- 20 Goeddel, D.V., Heyneker, H.L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D.G., Ross, M.J., Miozzari, G., Crea, R. and Seeburg, P.H. (1979) *Nature* 281: 544-548.
- Goodson, R.J. and Katre, N.V. (1990) *Biotechnology* 8: 343-346.
- Greenspan, F.S., Li, C.H., Simpson, M.E. and Evans, H.M. (1949) *Endocrinology* 45:455-463.
- Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. and Inouye, M. (1984) *EMBO J.* 3:2437-2442.
- 25 Hannum, C., Culpepper, J., Campbell, D., McClanahan, T., Zurawski, S. et al. (1994) *Nature* 368: 643-648
- Hershfield, M.S., Buckley, R.H., Greenberg, M.L. et al., (1987) *N.Engl. J. Medicine* 316: 589-596.
- Hill, C.P., Osslund, T.D. and Eisenberg, D. (1993) *Proc. Natl. Acad. Sci. USA* 90:5167-5171.
- 30 Horisberger, M.A. and Di Marco, S. (1995) *Pharmac. Ther.* 66: 507-534.
- Horoszewicz, J.S., Leong, S.S. and Carter, W.A. (1979) *Science* 206:1091-1093.
- Horton, R.M. (1993) in "Methods in Molecular Biology", White, B.A., ed. (Humana Press, Totawa, NJ), v.15, 214- 250.
- Hsiung, H.M., Mayne, N.G. and Becker, G.W. (1986) *Biotechnology* 4: 991-995.
- 35 Imai, N., Kawamura, A., Higuchi, M., Oh-eda, M., Orita, T., Kawaguchi, T., and Ochi, N. (1990) *J.Biochem.* 107:352-359.
- Innis, M.A., Gelfand, D.H, Sninsky, J.J. and White, T.J. eds. (1998) "PCR Protocols: A Guide to Methods and Applications" (Academic Press, San Diego, CA).

- Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S.D., Kaufman, R.J., Mufson, A., Seehra, J., Jones, S.S., Hewick, R., Fritsch, E.F., Kawakita, M., Shimizu, T. and Miyake, T. (1985) *Nature* 313: 806-810.
- 5 Johnson, D.L., Middleton, S.A., McMahon, F. Barbone, F.P., Kroon, D., Tsao, E., Lcc, W.H., Mulcahy, L.S. and Jolliffe, L.K. (1996) *Protein Expression Purif.* 7:104-113.
- Kadonaga, J., Gautier, A. Straus, D.R., Charles, A.D., Edge, M.D. and Knowles, J.R. (1984) *J. Biol. Chem.* 259: 2149-2154.
- Khan, F.R. and Rai, V.R. (1990) *Bioprocess Technology* 7:161-169.
- 10 Karasiewicz, R., Nalin, C. and Rosen, P. (1995) US Patent 5,382,657.
- Katre, N.V. (1990) *J. Immunology* 144: 209-213.
- Katre, N.V., Knauf, M.J. and Laird, W.J. (1987) *Proc. Natl. Acad. Sci. USA* 84: 1487, 1491.
- Kaufman, R.J. (1990) *Meth. Enzymol.* 185:537-566.
- Kingsley, D.M. (1994) *Genes Dev.* 8: 133-146.
- 15 Kinstler, O.B., Gabriel, N.E., Farrar, C.E. and DePrince, R.B. (1996) International Patent Application Number WO 96/11953.
- Knauf, M.J., Bell, D.P., Hirtzer, P., Luo, Z.-P., Young, P.D. and Katre, N.V. (1988) *J. Biol. Chem.* 263: 15064-15070.
- Komatsu, N., Nakauchi, H., Miwa, A., Ishihara, T., Eguchi, M., Moroi, M., Okada, M.,
- 20 Sato, Y., Wada, H., Yawata, Y., et al., (1991) *Cancer Research* 51: 341-348.
- Koshland, D. and Botstein, D. (1980) *Cell* 20: 749-760.
- Kutty G., Kutty, R.K., Samuel, W., Duncan, T., Jaworski, C., and Wiggert, B. (1998) *Biochem. Biophys. Res. Commun.* 246: 644-649.
- Lawton, L.N., Bonaldo, M.F., Jelenc, P.C., Qiu, L., Baumes, S.A., Marcellino, R.A.,
- 25 de Jesus, G.M., Wellington, S., Knowles, J.A., Warburton, D., Brown, S., and Soares, M.B. (1997) *Gene* 203: 17-26.
- Li, C.H. (1982) *Mol. Cell. Biochem.* 46: 31-41.
- Lin, F.-K., Suggs, S., Lin, C.-H., Browne, J.K., Smalling, R., Egrie, J.C., Chen, K.K., Fox, G.M., Martin, F., Stabinsky, Z., Badrawi, S.M., Lai, P.-H. and Goldwasser, E. *Proc. Natl. Acad. Sci. USA* 82: 7580-7584.
- 30 Lu, H.S., Boone, T.C., Souza, L.M., and Lai, P.H. (1989) *Arch. Biochem. Biophys.* 268: 81-92.
- Lucas, B.K., Giere, L.M., DeMarco, M.A., Shen, A., Chisolm, V., and Crowley, C.W. (1996) *Nucleic Acids Res.* 24:1774-1779.
- 35 Lydon, N.B., Favre, C., Bove, S., Neyret, O., Benureau, S., Levine, A.M., Scelig, G.F., Nagabhushan, T. L. and Trotta, P.P. (1985) *Biochemistry* 24: 4131-4141.
- MacGillivray, M.H., Baptista, J. and Johnson, A. (1996) *J. Clin. Endocrinol. Metab.* 81: 1806-1809.
- Maisano, F., Testori, S.A., and Grandi, G. (1989) *J. Chromatograph.* 472: 422-427.
- Mark, D.F., Lin, L.S. and Lu, S-D.Y. (1985) U.S. Patent 4,518,584.

- Mark, D.F., Lu, S.d., Creasey, A.a., Yamamoto, R. and Lin, L.S. (1984) *Proc. Natl. Acad. Sci. USA* 81: 5662-5666.
- Martal, J., Chene, N. and de la Llosa, P. (1985) *FEBS. Letts.* 180: 295-299.
- Martin, F.H., Suggs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, F.C., McNiece, I.K., Jacobsen, F.W., Mendiaz, E.A., Birkett, N.C. et al., (1990) *Cell* 63: 203-211.
- Massague, J. (1990) *Annu. Rev. Cell Biol.* 6: 597-641.
- Matthews, D.J., Topping, R.S., Cass, R.T. and Giebel, L.B. (1996) *Proc. Natl. Acad. Sci. USA* 93: 9471-9476.
- McDonald, N.Q. and Hendrickson, W.A. (1993) *Cell* 73: 421-424.
- 10 McKay, D.B. (1992) *Science* 257: 412.
- Meyers, F.J., Paradise, C., Scudder, S.A., Goodman, G. and Konrad, M. (1991) *Clin. Pharmacol. Ther.* 49: 307-313.
- Milburn, M.V., Hassell, A.M., Lambert, M.H., Jordan, S.R., Proudfoot, A.E., Graber, P. and Wells, T.N.C. (1993) *Nature* 363: 172-176.
- 15 Mills, J.B., Kostyo, J.L., Reagan, C.R., Wagner, S.A., Moseley, M.H. and Wilhelm, A.E. (1980) *Endocrinology* 107: 391-399.
- Mockridge, J.W., Aston, R., Morrell, D.J. and Holder, A.T. (1998) *Eur. J. Endocrin.* 138: 449-459.
- Monkarsh, S.P., Ma, Y., Aglione, A., Bailon, P. et al. (1997) *Anal. Biochem.* 247: 434- 440.
- Mordenti, J., Chen, S.A., Moore, J.A., Ferrailo, B.L. and Green, J.D. (1991) *Pharmacol. Res.* 8:1351-1359.
- 20 Morehead, H., Johnson, P.D. and Wetzal, R. (1984) *Biochemistry* 23: 2500-2507.
- Morioka-Fujimoto, K., Marumoto, R. and Fukuda, T. (1991) *J. Biol. Chem.* 266: 1728- 1732.
- Mott, H.R. and Campbell, I.D. (1995) *Current Opinion in Structural Biology* 5: 114-121.
- Ostermeier, M., De Sutter., K., Georgiou, G. (1996) *J. Biol. Chem.* 271: 10616-10622.
- 25 Paonessa, G., Graziani, R., de Serio, A., Savino, R., Ciapponi, L., Lahm, A., Ssalvati, A.L., Toniatti, C. and Ciliberto, G. (1995) *EMBO J.* 14: 1942-1951.
- Paul, W.E. ed. (1989) "Fundamental Immunology" (Raven Press, New York, NY).
- Pestka, S., Langer, J.A., Zoon, K.C. and Samuel, C.E. (1987) *Ann. Rev. Biochem.* 56: 727-777.
- Picken, R.N., Mazaitis, A.J., Maas, W.K., Rey, M. and Heyneker, H. (1983) *Infect. and Immun.* 42: 269-275.
- 30 Powers, R., Garrett, D.S., March, C.J., Frieden, E.A., Gronenborn, A.M. and Clore, G.M. (1992) *Science* 256: 1673- 1677.
- Ranjan, A. and Hasnain, S.E. (1995) *Virus Genes* 2:149-153.
- Redfield, C., Smith L.J., Boyd, J., Lawrence, G.M.P., Edwards, R.G., Smith, R.A.G., and Dobson, C.M. (1991) *Biochemistry* 30: 11029-11035.
- 35 Roitt, I.M., Brostoff, J., and Male, D.K. eds. (1989) "Immunology" (Gower Medical Publishers, New York, NY and London, UK)
- Rowlinson, S.W. Barnard, R., Bastiras, S., Robins, A.J., Brinkworth, R. and Waters, M.J. (1995) *J. Biol. Chem.* 270: 16833-16839.

- Shaw, G., Veldman, G. and Wooters, J.L. (1992) U.S. Patent 5,166,322.
- Sytkowski, A.J., Lunn, E.D., Davis, K.L., Feldman, L. and Siekman, S. (1998)
Proc. Natl. Acad. Sci. USA 95: 1184-1188.
- Tanaka, H., Satake-Ishikawa, R., Ishikawa, M., Matsuki, S. and Asano, K. (1991) Cancer
5 Research 51: 3710-3714.
- Teh, L.-C. and Chapman, G.E. (1988) Biochem. Biophys. Res. Comm. 150: 391-398.
- Thompson, S.A. (1992) J. Biol. Chem. 267: 2269-2273.
- Trill, J.J., Shatzman, A.R., and Ganguly, S. (1995) Curr. Opin. Biotechnol. 6:553-560.
- Tuma, R., Rosendahl, M. and Thomas, G. (1995) Biochem. 34: 15150-15156.
- 10 Urlaub, G. and Chasin, L.A. (1980) Proc. Natl. Acad. Sci. USA 77:4216-4220.
- Van Den Berg, C.L., Stroh, C., Hilsenbeck, S.G., Weng, C.-N., McDermott, M.J., Cox, G.N. and
Yee, D. (1997) Eur. J. Cancer 33: 1108-1113.
- Voss, T., Falkner, E., Ahorn, H., Krystek, E., Maurer-Fogy, I., Bodo, G., Hauptmann, R.
(1994) Biochem. J. 298, 719-725.
- 15 Walter, M.R., Cook, W.J., Ealick, S.E., Nagabhusan, T.L., Trotta, P.T. and Bugg, C.E. (1992)
J.Mol. Biol. 224: 1075-1085.
- Wang, G.L. and Semenza, G. L. (1993) Blood 82:3610-3615.
- White, B. A. (1993) in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current
Methods and Applications edited by Humana Press, Inc. , Totowa,NJ.
- 20 Wojchowski, D.M., Orkin, S.H. and Sytkowski, A.J. (1987) Biochim. Biophys. Acta 910: 224-232.
- Wrighton, N.C., Farrell, F.X., Chang, R. et al., (1996) Science 273: 458-463.
- Yamaoka, T., Tabata, Y. and Ikada, Y. (1994) J. Pharm. Sci. 83: 601-606.
- Baldwin, M.D., Zhou, X.J., Ing, T.S. and Vaziri, N.D. (1998) ASAIO J. 44: 44-47.
- Balkwill, F.R., Goldstein, L. and Stebbing, N. (1985) Int. J. Cancer 35: 613-617.
- 25 Balkwill, F.R. (1986) Methods Enzymology 119: 649-657.
- Cecil, R. and McPhee, J.R. (1959). Advances in Protein Chemistry 14, 255-389.
- Cox, G.N., McDermott, M.J., Merkel, E., Stroh, C.A., Ko, S.C., Squires, C.H., Gleason, T.M. and
Russell, D. (1994) Endocrinology 135: 1913-1920.
- Greenspan, F.S., Li., C.H., Simpson, M.E. and Evans, H.M. (1949) Endocrinology 45: 455-463.
- 30 Johns, T.G., Mackay, I.R., Callister, K.A., Hertzog, P.J., Devenish, R.J. and Linnane, A.W.
(1992) J. Natl. Cancer Institute 84: 1185-1190.
- Lindner, D.J. and Borden, E.C. (1997) J. Interferon and Cytokine Research 17:681-693.
- Matsumoto, T., Endoh, K., Kamisango, K., Akamatsu, K., Koizumi, K., Higuchi, M., Imai, N.,
Misui, H. and Kawaguchi, T. (1990) Br. J. Haematol. 75: 463-468.
- 35 Torchinskii, Y.M. (1971) in "Sulfhydryl and Disulfide Groups of Proteins" in Studies of Soviet
Science (1971) Nauka Press, Moscow.
- Trotta, P.B. (1986) Seminars in Oncology XIII Supplement 2: 3-12.
- Vaziri, N.D., Zhou, X.J., and Liao, S.Y. (1994) Am. J. Physiol. 266: F360-366.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention.

Claims:

1. A method for obtaining a soluble protein having a free cysteine comprising the step of:
 - (a) obtaining a host cell capable of expressing the soluble protein;
 - (b) exposing the host cell to a cysteine blocking agent; and
 - (c) isolating the soluble protein from the host cell.
2. The method of claim 1, further comprising disrupting the host cell in the presence of the cysteine blocking agent and isolating the protein from the soluble fraction of the disrupted host cell.
3. The method of claim 1, wherein exposing the host cell to a cysteine blocking agent occurs before, during or after synthesis of the soluble protein by the host cell and wherein the soluble protein is secreted from the host cell.
4. The method of claims 1 to 3, wherein said host cell is a bacteria, yeast, insect or mammalian cell.
5. The method of claims 1 to 3, wherein said host cell is a bacteria cell.
6. The method of claim 5, wherein said host cell is *E.coli*.
7. The method of claims 1 to 3, wherein said soluble protein is a recombinant protein.
8. The method of claim 7, wherein said recombinant protein is a cysteine mutein of a member of the growth hormone supergene family, a derivative or an antagonist thereof.
9. The method of claim 8, wherein said member is growth hormone.
10. The method of claim 8, wherein said member is erythropoietin.
11. The method of claim 8, wherein said interferon is alpha interferon alpha (IFN- α).
12. The method of claim 8, wherein said alpha interferon is interferon alpha 2 (IFN- α 2).
13. The method of claim 7, wherein said recombinant protein is a cysteine mutein of a member of the TGF-beta superfamily, platelet derived growth factor-A, platelet derived growth factor-B, nerve growth factor, brain derived neurotrophic factor, neurotrophin-3, neurotrophin-4, vascular endothelial growth factor, or a derivative or an antagonist thereof.

14. The method of claim 7, wherein said recombinant protein is a cysteine mutein of a heavy or light chain of an immunoglobulin or a derivative thereof.

15. The method of claim 1, wherein said cysteine blocking agent is a thiol-reactive compound.

5

16. The method of claim 15, wherein said thiol-reactive compound is cystine, cystamine, dithioglycolic acid, oxidized glutathione, iodine, hydrogen peroxide, dihydroascorbic acid, tetrathionate, O-iodosobenzoate or oxygen in the presence of a metal ion.

10

17. The method of claim 15, wherein said thiol-reactive compound is cystine.

18. The method of claims 1 to 3, further comprising attaching a cysteine-reactive moiety to said isolated protein to form a cysteine modified protein.

15

19. The method of claims 1 to 3, further comprising attaching a polyethylene glycol to said isolated protein to form a pegylated protein.

20. A pegylated human growth hormone (hGH) or a derivative thereof having an EC_{50} of less than about 110 ng/ml.

20

21. The pegylated hGH of claim 20, wherein the PEG moiety is attached to a C-D loop or a region proximal to Helix A of said hGH.

22. A pegylated erythropoietin (EPO) or a derivative thereof having an EC_{50} of less than about 1000 ng/ml.

25

23. The pegylated EPO of claim 22, wherein the PEG moiety is attached to a C-D loop or an A-B loop of EPO.

24. A pegylated alpha interferon (IFN- α 2) or a derivative having an EC_{50} of less than about 100 pg/ml.

30

25. The pegylated IFN- α 2 of claim 24, wherein the PEG moiety is attached to a region proximal to Helix A or a C-D loop of IFN- α 2.

35

26. A multimeric protein comprising at least two proteins each having a free cysteine and wherein said proteins are attached through said free cysteines.

27. A method of treating a condition treatable with growth hormone, EPO or an alpha interferon, comprising administering to a patient in need thereof an effective amount of a cysteine variant of growth hormone, EPO or alpha interferon, or a derivative thereof to treat said condition.

- 5 28. The method of claim 27, wherein said derivative is a pegylated cysteine variant of growth hormone, EPO or alpha interferon.

29. A method for covalently modifying a protein produced according to claims 1 to 3, comprising the steps of:

- 10 (a) purifying the soluble protein;
 (b) reducing the protein with a disulfide-reducing agent; and
 (c) exposing the protein to a cysteine-reactive moiety to obtain a cysteine-modified protein.

- 15 30. The method of claim 29, further comprising isolating the cysteine-modified protein from the unmodified protein.

31. The method of claim 29, wherein the cysteine-reactive moiety is a polyethylene glycol.

1/1

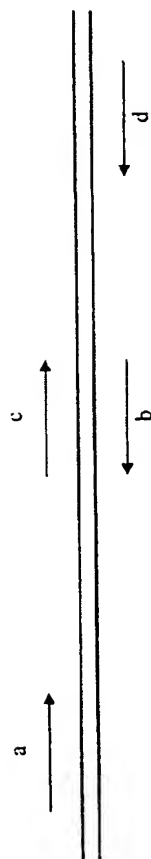


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/00931

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/00, 15/20; C07K 17/08, 14/555, 14/475

US CL : 435/69.51, 471, 252.3, 252.8, 255.1; 530/402, 351

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.51, 471, 252.3, 252.8, 255.1; 530/402, 351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 95/32003 A1 (AMGEN BOULDER INC.) 30 Novmeber 1995 (30.11.95), especially see, page 6, lines 2-10, page 24, lines 7-30, page 29, lines 28-30 and claims 1, 2.	1-9, 15-16, 18-19, 26, 29-31 ----- 10-14, 17, 20-25, 27-28
X --- Y	US 5,766,897 A (BRAXTON) 16 June 1998 (16.06.98), see especially abstract and column 11, lines 39-65, column 12 lines 1-6, table 1A on column 12, column 37, lines 1-5, and column 47, lines 3-14.	1-13, 15, 18-19, 26-31 ----- 14, 16-17, 20-25



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 MARCH 2000

Date of mailing of the international search report

26 APR 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized Officer

FOZIA HAMUD

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00931

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,206,344 A (KATRE et al.) 27 April 1993 (27.04.93), see especially abstract and column 12, lines 50-68, Example 1 on column 16, claims 1, 9.	1-8, 15-16, 18-19, 26, 29-31 ----- 9-14, 17, 20-25, 27-28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/00931

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

East, US pat full; STN via medline, caplus, embase; search terms: cysteine muteins, free cysteine; (glutathione, cystine, pyridine disulfides, cysteine blocking agents), PEG or polyethylene glycol,